Cardioprotective Potential of Ischemic and Anesthetic Pre- and Postconditioning in Remodeled Rat Hearts

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Thesis

A. Abstract

Objectives of the studies were to test (1) whether ischemic postconditioning (IPostC) is protective in post-myocardial infarct (MI)-remodelled and one-kidney one-clip (1K1C) hypertensive rat hearts, (2) whether isoflurane-induced late cardioprotection is preserved in post-infarct remodelled hearts, (3) whether volatile anesthetics also provide protection in various clinical scenarios. We found that (1) IPostC prevented ischemia/reperfusion injury and improved cardiac function in both diseased heart models. The protection was abolished by PI3K inhibition. Western blot analysis and in vitro kinase assays identified protein kinase B (PKB)/Akt but not extracellular-signal regulated kinase 1/2 (ERK1/2) pathway as the main reperfusion injury salvage kinase pathway in this protection. (2) Isoflurane-induced late protection was preserved, but substantially shorter in post-infarct remodelled rat hearts. The protection was blocked by cyclooxygenase-2 inhibition. Impaired CREB activation and an increased level of the CREB antagonist ICER were associated with impaired cyclooxygenase-2 activity. Thus a shorter second window of protection occurs in remodelled hearts. In patients and healthy volunteers, volatile anesthetics favourably modulated gene expression and elicited anti-inflammatory and endothelial protective effects.

Conclusions: (1) IPostC can protect hypertrophied hearts against I/R injury in a PI3K dependent way. (2) Isoflurane fails to mobilize cyclooxygenase-2-inducing CREB in remodelled hearts, which is associated with a shortening of the second window of protection. Therefore, volatile anesthetics are promising drugs potentially providing multi-organ protection.

A. Zusammenfassung

Ziel der Studien war es (1) zu testen, ob ischämische Postkonditionierung (IPostC) auch protektiv wirkt in Herzen mit altem Herzinfarkt und hypertensiv-veränderten Herzen mit Hypertrophie, (2) zu testen, ob der Isofluran-induzierte Herzschutz auch in Infarkt-remodellierten Herzen effektiv ist, (3) zu testen, ob volatile Anästhetika auch am Patienten unter klinischen Bedingungen eine Schutzwirkung entfalten können. Unsere Studien zeigen, dass (1) IPostC den Ischämie/Reperfusionsschaden auch in Infarkt Herzen und hypertensiv-veränderten Herzen deutlich vermindert. Dieser Schutz kann durch Hemmung der PI3K aufgehoben werden. Western blot Analysen und in vitro Kinase Assays zeigen, dass die Protein Kinase B nicht aber die extracellular-signal regulated Kinase (ERK1/2) eine wichtige Rolle spielt. (2) dass das „zweite“ oder späte Fenster des Isofluran-induzierten Herzschutzes zwar vorhanden ist in kranken Herzen, doch deutlich kürzer ist als in gesunden Herzen. Der Schutz kann komplett mit Cyclooxygenase-2 Inhibitoren aufgehben werden. Bei der Verkürzung des protektiven Fensters spielt eine verminderte Mobilisierung von CREB in den ICER-überexprimierenden Infarkt Herzen eine Rolle. In Patienten und gesunden Probanden, modulieren volatile Anästhetika die Genexpression günstig und wirken dadurch entzündungshemmend und Endothel-schützend.

Schlussfolgerungen: (1) IPostC schützt hypertrophierte Herzen gegen Ischämie/Reperfusionsschaden via PI3K-PKB Signalweg. (2) In kranken Herzen hat Isofluran eine kürzere Schutzwirkung. Volatile Anästhetika scheinen daher vielversprechende Medikamente in der Therapie lebenswichtiger Organe zu sein, auch wenn sie vorgeschädigt sind.

B. Background

Ischemia-reperfusion (I/R) injury

For patients with an acute myocardial ischemia, clinical intervention like coronary artery bypass grafting is necessary to restore the blood flow and thus recover cardiac function. Observations on perioperative patients revealed that there is still some injury despite the timely treatment with clinical interventions. This is caused by the restoration of blood flow following ischemia and is called I/R injury [1].

Biochemical and cellular changes during ischemia and reperfusion (see Fig.1)

Decreased ATP generation and acidosis:

Ischemia reduces blood flow to related tissue which causes not only a decreased oxygen supply but also the loss of washout of toxic metabolites from the respective tissue. With the loss of oxygen supply, oxidative phosphorylation stops and anaerobic glycolysis increases as a compensation, leading to a decrease of ATP production. At the same time, lactic acid accumulates and intracellular pH (pH_i) decreases because of loss of efficient washout of metabolites [1,2].

Accumulation of intracellular and mitochondrial calcium:

In cardiomyocytes, cytosolic Ca^{2+} is usually maintained at a relatively low level (10-100 nM) by transporting Ca^{2+} out of the cell and to the cisternae of the sarcoplasmic reticulum (SR), and by protein binding. Under physiological conditions, cytosolic Ca^{2+} increases by entry through plasma membrane Ca^{2+} channels, or release of Ca^{2+} from the SR via binding of Ca^{2+} to the ryanodine receptor (RyR) or via binding of inositol trisphosphate (IP3) to the inositol trisphosphate receptor (IP3R). Intracellular Ca^{2+} is pumped out of the cell via the plasma membrane calcium ATPase (PMCA) and the Na^+/Ca^{2+} exchanger. The sarco-endoplasmic reticulum calcium ATPase (SERCA) transports Ca^{2+} into the SR. Mitochondria can also buffer Ca^{2+} by transporting the ion into the mitochondrial matrix via Ca^{2+} uniporters or out of mitochondria via Na^+/Ca^{2+} exchangers [3]. During ischemia, an increased amount of lactic acid and the decreased intracellular pH activate the Na^+-H^+ exchanger. As a result, the intracellular sodium concentration $[Na^+]_i$ increases. The low ATP level leads to a decrease in activity of the ATP consuming Na^+-K^+-pump which further increases the intracellular

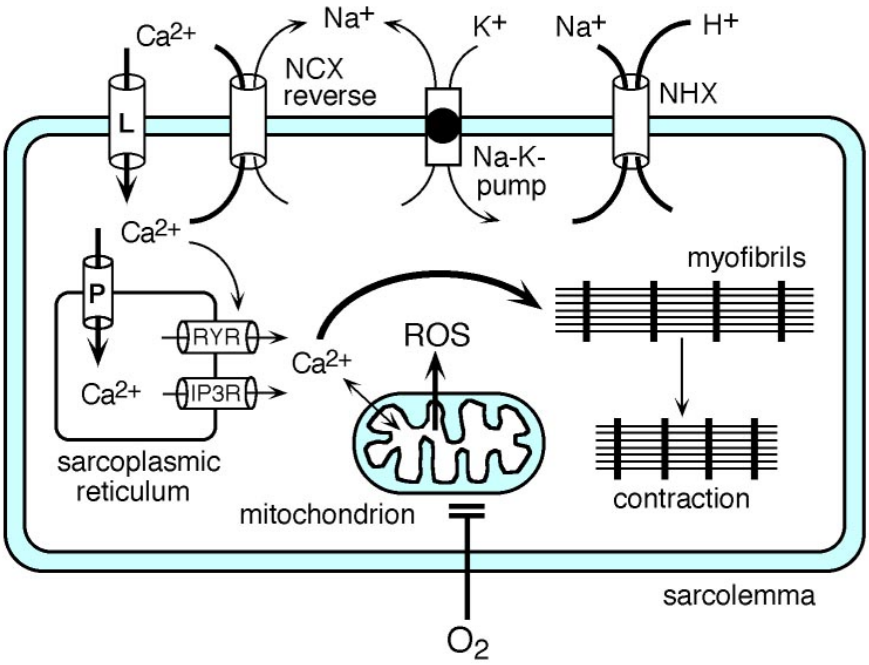
accumulation of sodium. The resultant lower outside-inside sodium gradient causes the Na^+-Ca^{2+} exchanger to work in the reverse mode, pumping Na^+ outside and Ca^{2+} into the cell [4]. Ca^{2+} also enters the cell via the L-type Ca^{2+} channels. On the other hand, a decrease in ATP results in reduced activities of the Ca^{2+} pump in the plasma membrane and of the calcium ATPase (SERCA) in the sarcoplasmic reticulum. All these events lead to an increased cytosolic Ca^{2+}. Mitochondrial Na^+/Ca^{2+} exchangers operate in the reverse way, leading to an increased mitochondrial calcium Ca^{2+} [3].

Generation of reactive oxygen species (ROS):

In oxidative phosphorylation, oxygen is consumed and finally reduced to H_2O. Most of the oxygen is reduced to H_2O by the mitochondrial electron transport. While a small part of oxygen is partially oxidized to toxic reactive oxygen species. Under normal conditions, cells or tissues can tolerate small amounts of ROS and reduce ROS to H_2O. However, under ischemia, this balance is destroyed, and during reperfusion, there is a burst of ROS generation [5].

Fig.1

A. Ischemia



B. Reprfusion

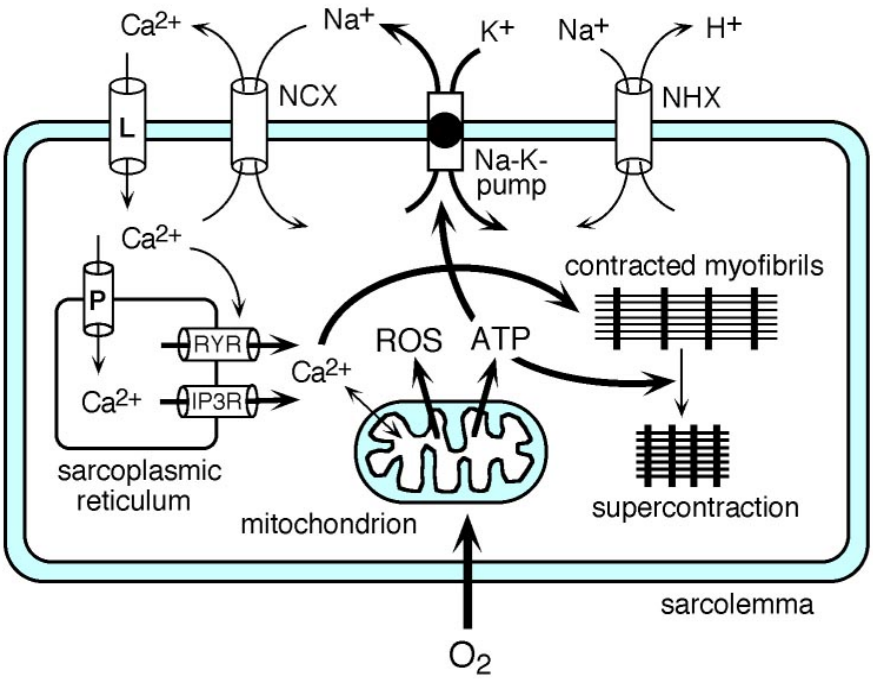


Fig.1 Changes and consequences of cation fluxes during ischemia reperfusion: (A) Cessation of oxygen supply in ischemia leads to a loss of ATP production and an increase of reactive oxygen species (ROS) in the mitochondria. Reduced activity of the ATP consuming Na^+-K^+-pump lowers the outside-inside sodium gradient, Na^+ accumulates in the myocyte and the resting membrane potential is lowered. With the development of acidosis, the Na^+-H^+-exchanger (NHX) further increases intracellular Na+. Under these conditions the Na^+-Ca^{2+}-exchanger (NCX) operates in the reverse mode, letting Ca^{2+} into the cell. Ca^{2+} also enters through the sarcolemmal L-type voltage-gated Ca^{2+}-channel (L) as the resting membrane potential is low. The increased Ca^{2+} is taken up into the sarcoplasmic reticulum (SR) by the SR Ca^{2+}-pump SERCA2 (P) and released from there via two types of release channels, the ryanodine receptor channel (RYR) and the IP3 receptor channel (IP3R), leading to contraction. (B) Reoxygenation during reperfusion restores ATP production with a further boost of ROS. Reactivation of the Na^+-K^+-pump by ATP slowly restores the sodium gradient leading to normal cation fluxes with the NCX eventually extruding the excess of cytosolic Ca^{2+}. During the early reperfusion phase when the intracellular Ca^{2+} level is still high, myocardial contracture (supercontraction of myocytes) may develop [Adopted from [4]].

Mitochondrial permeability transition (MPT):

Mitochondria are the key determinants of cell survival and death under different conditions. Under normal (aerobic) conditions, the electron transport generates an electrochemical gradient across the inner mitochondrial membrane (IM), leading to the membrane potential $\Delta\varphi_m$ ($\approx$ -200 mM) and the proton gradient (ΔpH). The electrochemical gradient is critical for the ATP synthase to phosphorylate ADP to ATP. The maintenance of this gradient needs the IM to be relatively impermeable to ions. On the other hand, the mitochondrial permeability transition (MPT) [which is caused by the opening of mitochondria permeability transition pore (mPTP)] may lead to cell death and may play a critical role in I/R injury [6]. mPTP is sensitive to high concentrations of Ca^{2+} and Pi, ROS, the depletion of ATP, and high pH. During ischemia, the accumulation of intracellular calcium, long-chain fatty acids, as well as ROS increase mitochondrial susceptibility to MPT. The mPTP remains closed during ischemia, possibly due to acidosis, elevated Mg^{2+} and depressed electron transport. At reperfusion, acidosis is corrected, leading to increased cellular pH. Normalized pH together with a further influx of calcium into the mitochondria and a burst of ROS lead to the uncontrolled opening of the mPTP [6], that is, MPT. MPT converts mitochondria from ATP producers to ATP consumers, accelerating cellular energy depletion and hasting cell death. mPTP opening also leads to matrix swelling, outer membrane rupture, release of apoptotic signalling molecules such as cytochrome c from the intermembrane space, and irreversible mitochondrial injury. Hausenloy et al.. showed for the first time that protection can be obtained by treatment with cyclosporin A, an inhibitor of mPTP, if administrated at the beginning of reperfusion [7]. Many other studies investigating pre-and postconditioning showed that protective signalling pathways converge at the mitochondria level, especially the mPTP, both during pre- and postconditioning [8,9].

Myocardial responses to acute ischemia

The contractility of the cardiac tissues markedly reduced only a few seconds after the onset of ischemia because of decreased ATP content. Within minutes, intracellular acidosis develops, and Cellular Ca^{2+} concentration increases and cell swelling begins. Then cells undergo irreversible injury and cellular ultra-structure changes, which is followed by the release of cytoplasmic proteins such as troponins and CK-

MB [4]. Myocardial ischemia followed by reperfusion causes myocardial stunning or hibernation despite restoration of blood flow. Myocardial stunning is a reversible damage which is characterized by low contractility despite high coronary flow. Myocardial hibernation is an irreversible damage in which ventricular contractility is diminished as a consequence of reduced coronary blood flow [4]. This leads to the metabolic remodelling of the tissue.

Cardioprotection against I/R injury (see Fig.2)

Myocardial preconditioning

For decades, research efforts have been made to develop measures to alleviate or eliminate I/R injury. Murry et al. first reported that transient nonlethal episodes of ischemia conferred protection against a subsequent sustained episode of potentially lethal myocardial ischemia, reducing infarct size to 70-80% when compared to non-preconditioned hearts. This is called ischemic preconditioning (IPC) [10]. Since then, pharmacological agents were also shown to be able to mimic IPC. Among them, volatile anesthetics were found to be very effective [11].

Myocardial postconditioning

Recently, an alternative approach to cardioprotection was reported by Zhao et al. [12]. This consists of a short series of repetitive cycles of brief reperfusion and re-occlusion of the coronary artery applied immediately at the onset of reperfusion, and is termed “postconditioning” (IPostC). Studies indicated that recruitment and activation of the prosurvival kinases, i.e. phosphatidylinositol-3-OH kinase (PI3K)-Akt and the p42/p44 extracellular signal regulated kinases (ERK1/2), at the time of reperfusion, contribute to cardioprotection of both ischemic preconditioning and ischemic postconditioning. These prosurvival kinases are collectively called the *reperfusion injury salvage kinases* (RISKs) [13]. We and other research groups found that pharmacological postconditioning (i.e. by volatile anesthetics) could induce cardioprotection during early reperfusion after myocardial ischemia through activation of PI3K-PKB/Akt pathway [14,15]. Detailed mechanisms of pre- and postconditioning will be discussed later.

Fig.2

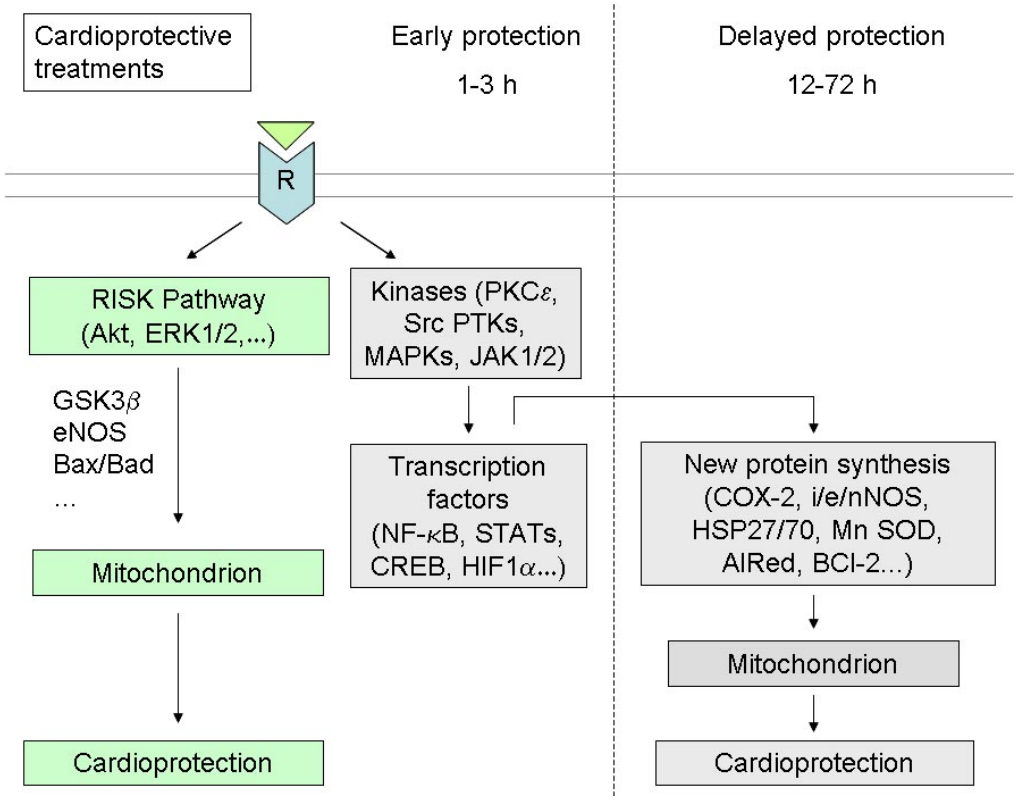


Fig.2 Signalling for cardiac preconditioning: The signalling components depicted illustrate current knowledge regarding the mechanisms of early (left of dashed line) and delayed (right of dashed line) cardiac preconditioning. AlRed=aldose reductase; BCl-2=B-cell lymphoma 2; Bax=BCL2-associated X protein; Bad=proapoptic protein Bad; COX-2=cyclooxygenase type 2; CREB=cAMP response element binding protein; ERK1/2=extracellular signal regulated kinase 1/2; GSK3β=glycogen synthase kinase 3β; HIF1α=hypoxia-inducible factor 1α; HSP 27/70=heat shock protein 27/70; i/e/nNOS=inducible/endothelia/neuro nitric oxide synthase; JAK=janus kinase; MAPKs=mitogen activated protein kinases; NF-κB=nuclear factor-κB; PKC=Protein kinase C; STATs=signal transducers and activators of transcription; Src PTK=Src protein tyrosine kinase; MnSOD=manganese superoxide dismutase; R=receptors.

Late or delayed preconditioning

Cardiac protective treatments elicited by preconditioning can only protect for 1-3 hours [16]. In 1993, Marber and his colleagues and Kuzuya and colleagues found that there is a second window of protection (also called late preconditioning), which occurs at about 12-24 hours after the cardioprotective treatments and lasts for up to 72 hours [17,18]. Physical stress (i.e. ischemia, heat stress, exercise) cause release of chemical signals [i.e. nitric oxide (NO), ROS, adenosine] that serve as triggers for the development of late preconditioning. Pharmacological stimuli (i.e. endotoxin and its derivatives, cytokines, ROS, NO donors, adenosine A1 or A2 agonists, K_{ATP} channel openers, delta opioid receptor agonists, alpha adrenergic receptor agonists) can also induce late preconditioning. These stimuli recruit the activation of different kinases [i.e. protein kinase C ε (PKCε), Src protein tyrosine kinases (Src PTKs), mitogen activated protein kinases (MAPKs), janus kinase 1/2 (JAK1/2)], leading to activation of transcription factors such as nuclear factor-κB (NF-κB), signal transducers and activators of transcription (STATs), hypoxia-inducible factor 1α (HIF1α), and cAMP response element binding protein (CREB), resulting in increased transcription and synthesis of cardioprotective proteins. Cyclooxygenase 2 (COX-2) is a rate limiting enzyme of prostaglandins, among which PGE2 and PGI2 are likely effectors of COX-2 mediated cardiac protection [19]. NO appears to act as both trigger and mediator of late preconditioning. All isoforms of NOS (iNOS, eNOS, and nNOS) are considered to be involved in late preconditioning [20,21]. There are also some other proteins involved in the second window of protection, including aldose reductase (AlRed), Bcl-2, heat shock protein (HSP) 27/70, manganese superoxide dismutase (Mn SOD) [22].

Mechanisms of cardioprotection underlying pre- and postconditioning

General remarks

As mentioned above, multiple studies demonstrated that pre- or postconditioning converge on the RISK pathways at the time of myocardial reperfusion. The key members of the RISK pathways are: Akt/PKB, ERK1/2, and PKC-ε and their downstream targets. More and more observations results indicate that the mPTPs is the final target of the RISK pathways [23]. As mPTP opens at the very beginning of

reperfusion, the various treatment options must be applied before ischemia or at the onset of reperfusion to protect the heart against I/R injury. mPTP inhibitors applied several minutes after the onset of reperfusion failed to protect the heart against I/R injury [24].

Ischemic pre- and postconditioning **(see Fig.3)**

Ischemic preconditioning can cause the release of endogenous substances such as adenosine, bradykinin, norepinephrine, and opioids. These substances work as triggers of preconditioning, leading to activation of a number of signalling cascades via G-protein coupled receptors (GPCRs) [4].

Role of adenosine:

Adenosine is the best studied endogenous trigger that can protect against I/R injury. There are four adenosine receptor subtypes (A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR) [25]. A_1ARs and A_{2A}ARs are expressed in adult ventricular myocytes. Whether functional A_3 or A_{2B}AR receptors are expressed in these cells is not clear yet. Their activation reduces the level of reactive oxygen species and attenuates stunning in rat ventricular myocytes [26]. Binding of adenosine to A_1AR leads to activation of several kinase pathways, including PKC, MAPKs, and PI3K/Akt [27-29]. These signalling pathways converge on mitochondrial targets such as mPTP and mK_{ATP} channels [7]. Cardioprotection induced by IPC can be abolished by administration of a non-specific adenosine receptor blocker during reperfusion [30], indicating an important role of ligand-receptor binding in IPC. Additional studies revealed that this binding in IPostC is also required at the time of reperfusion [31-33].

Role of bradykinin:

Bradykinin is a vasodilator. A short preconditioning period of ischemia leads to a marked increase in tissue and plasma bradykinin levels [34]. Bradykinin B2 receptor blockers can abolish protection from IPC in rabbit hearts [35] and isolated rat hearts [36]. Yang et al. showed that IPC did not confer cardioprotection in B2 receptor knock-out mice [37].

Fig.3

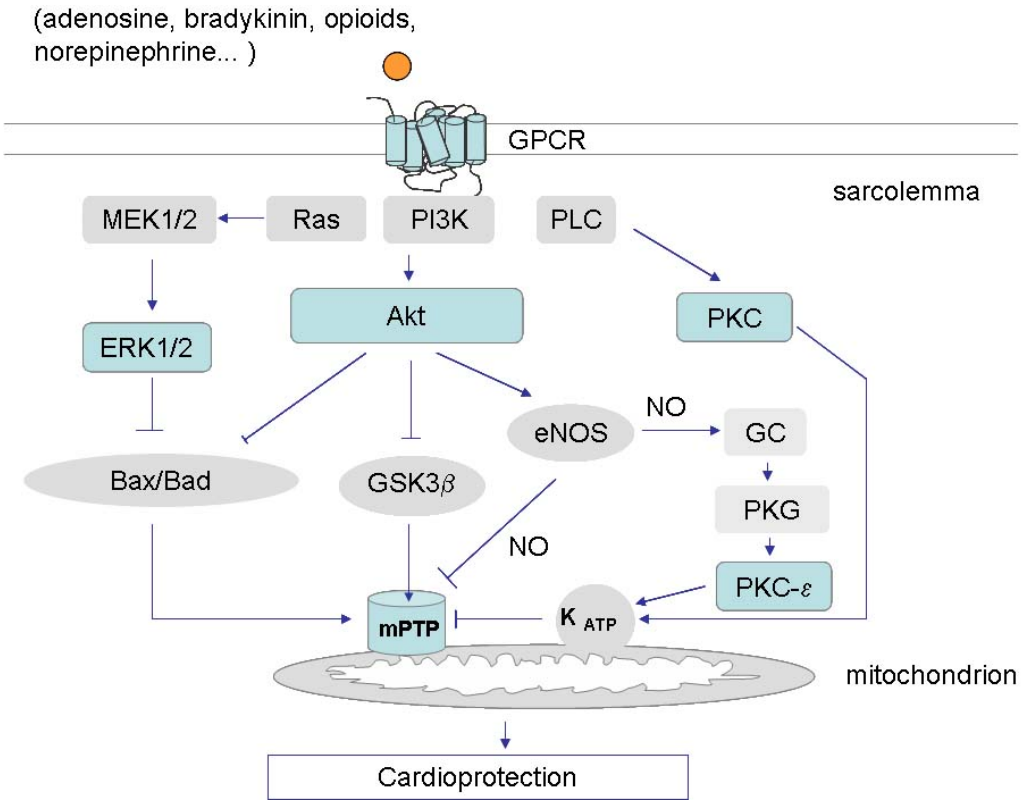


Fig.3 Signaling pathways involved in ischemic pre- and postconditioning: Ischemic pre- or postconditioning treatments induce release of intrinsic triggers of cardioprotection such as adenosine, bradykinin, opioids, and norepinephrine. These triggers activate GPCRs, leading to activation of RISK signalling pathways like ERK1/2 pathway, Akt pathway, and/or PKC pathway. These pathways converge on the mPTPs to have their cardioprotective effects via several mediators: GSK3β, Bax/Bad, eNOS, and mK_{ATP}. Arrows indicate positive actation and lines with blunted ends (-) indicate inhibition. GPCR=G protein-coupled receptor; MEK1/2=mitogen activated kinase kinase 1/2; ERK1/2=extracellular signal regulated kinase 1/2; PI3K= phosphatidylinositol-3-OH kinase; PLC=phospholipases C; PKC=protein kinase C; Bax=BCL2-associated X protein; Bad=proapoptic protein Bad; GSK3β=glycogen synthase kinase 3β; NO=nitric oxide; eNOS=endothelial NO synthase; GC=guanylate cyclise; PKG=protein kinase G; mK_{ATP}=mitochondrial ATP dependent potassium channel.

Role of opioids:

It has been shown that opiod receptor ligands such as morphine and fentanyl can mimic IPC [38,39]. Administration of δ-opioid receptor antagonists abolished IPC [40].

Role of norepinephrine:

Administration of exogenous α-adrenoceptor agonist norepinephrine mimics preconditioning [41]. And this protection can be blocked by a α-adrenenic blocker.

RISK pathways:

In 2004, Tsang Andrew and his colleagues found that activation of PI3K-Akt signalling pathway is required for IPostC in isolated perfused rat heart[42]. In 2005, additional work from the same group found that IPC induces phosphorylation of Akt and/or ERK-1/2 during both the preconditioning and the reperfusion phase in isolated perfused rat hearts. Inhibition of phosphorylation of these kinases at the time of reperfusion abolished the cardioprotection elicited by IPC [43]. Some other studies further confirmed the important role of PI3K/Akt and ERK-1/2 in IPC and IPostC [33,44,45]. The role of PKC in IPC induced cardioprotection is controversial. There are several subtypes of PKC. PKC-α, PKC-δ, and PKC-ε are expressed in the rat heart, and PKC-ε and PKC-η in the rabbit heart [46]. In some studies, PKC-δ and PKC-ε were found to have opposite roles in cardioprotection. In isolated rat heart, administration of φεRACK, a specific PKC-ε activator, mimics ischemic preconditioning. Administration of δV1-1, a selective PKC-δ inhibitor, prevents reperfusion injury [47]. One study indicates that PKC-ε isoform is an important member of the survival kinases in IPostC [48].

End effectors:

Evidence is accumulating that all RISK pathways converge on mPTP, preventing its opening, and thus alleviating I/R injury [2]. The possible mechanisms through which the RISK pathways inhibit mPTP opening include: (a) glycogen synthase kinase 3β (GSK-3β): GSK-3β can phosphorylate VDAC and disrupt the binding of voltage dependent anion channel (VDAC) to Hexokinase II, leading to mPTP opening [49,50]. Phosphorylation of GSK-3β at Serine 9 by Akt inhibits its activity and thus prevents

mPTP opening. (b) eNOS: On one hand, eNOS induces the generation of nitric oxide, which can suppress mPTP opening [51]. On the other hand, eNOS can work through the PKG-PKC-ε-mK_{ATP} pathway, leading to the opening of mK_{ATP} channel [52]. (c) Inhibition of pro-apoptotic proteins BAX/BAD [53,54]. (d) Opening of mK_{ATP} channel can also prevent mPTP opening [55].

Anesthetics induced pre- or postconditioning **(see Fig.4)**

In 1976, Bland JH and his colleagues found for the first time that halothane protected the non-failing canine heart against experimental myocardial ischemia [56]. Later on, many anesthetics (i.e. isoflurane, enflurane, sevoflurane, desflurane, opioids, ethanol) were found to have similar effects mimicking IPC [11]. Volatile anesthetics are the most frequently used preconditioning drugs because they are used for general anesthesia. Till now, little is known about how volatile anesthetics work in the brain and other tissues and whether they have specific receptors or not. However, studies found that PKC activation, mK_{ATP} channels, and probably activation of G-protein coupled receptors (i.e. A1 adenosine receptor, adrenergic receptors) are involved in volatile anesthetics induced preconditioning [11]. Cope DK [57] found that, 8-sulfophenyl theophylline, a non-specific adenosine receptor blocker, and chelerythrine, a specific PKC blocker, can inhibit halothane induced preconditioning in an in vivo rabbit model. Later on, studies reported that isoflurane induced preconditioning was inhibited by a non-specific adenosine receptor blocker in an in vivo rabbit mode, and was inhibited by an adenosine 1-specific receptor blocker in a dog model of regional ischemia. Desoflurane-induced preconditioning was reported to be inhibited by α-adrenergic blocker phentolamine, and, β-adrenergic blocker propranolol [58]. PKC was also found to be involved in the protective effect of isoflurane against stunning [39]. Using blockers of the sarcolemmal and mitochondrial K_{ATP} channels, studies indicate the involvement of both sK_{ATP} and mK_{ATP} channels in volatile-anesthetic induced preconditioning [11].

Chiari PC and his colleagues [14] reported for the first time that, isoflurane, a volatile anesthetic, can induce postconditioning in rabbit hearts when applied at the early stage of reperfusion. This protection is dependent on the activation of PI3K-Akt signalling pathway. In the same year, Feng J and his colleagues reported that isoflurane can induce postconditioning in rat hearts through inhibiting opening of the

mPTP [15]. Inhibition of GSK-3β, a downstream target of PI3K-Akt pathway, might be involved.

Fig.4

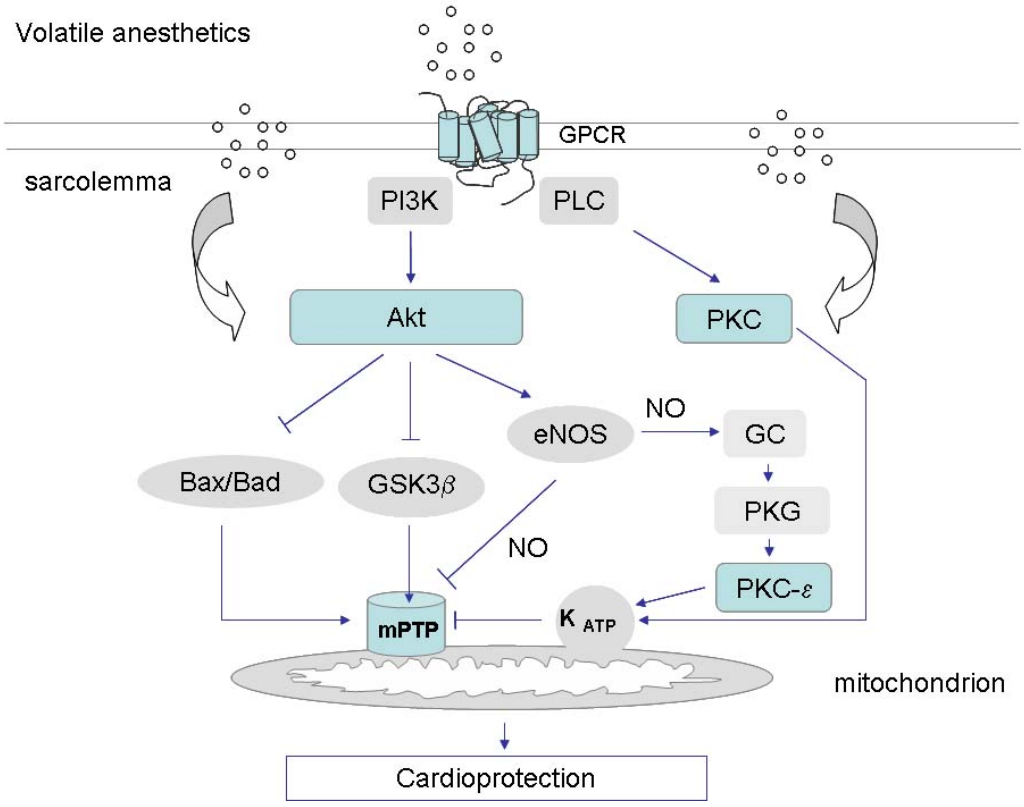


Fig.4 Signaling pathways involved in volatile anesthetic induced pre- and postconditioning: Volatile anesthetics activate RISK signalling pathways like Akt pathway, and/or PKC pathway through GPCRs or some unknown mechanisms. These pathways converge on the mPTPs to have their cardioprotective effects via several mediators: GSK3β, Bax/Bad, eNOS, and mK_{ATP}. Arrows indicate positive activation and lines with blunted ends (-) indicate inhibition. GPCR=G protein-coupled receptor; PI3K=phosphatidylinositol-3-OH kinase; PLC=phospholipases C; PKC=protein kinase C; Bax=BCL2-associated X protein; Bad=proapoptic protein Bad; GSK3β=glycogen synthase kinase 3β; NO=nitric oxide; eNOS=endothelial NO synthase; GC=guanylate cyclise; PKG=protein kinase G; PKCε=protein kinase C-ε; mK_{ATP}=mitochondrial ATP dependent potassium channel.

Other pharmacological mimics of pre- and postconditioning

Besides ischemic stimuli and anesthetics, there are many other pharmacological agents that are able to mimic pre- or postconditioning. Mostly, they are G-protein coupled receptor ligands (i.e. adenosine, bradykinin, opioids, adrenomedullin, glucagon like peptide 1, adrenomedullin, and urocortin); growth factors (i.e. transforming growth factor β-1, insulin, insulin like growth factor-1, and corticotrophin-1); natriuretic peptides (atrial natriuretic peptide and brain natriuretic peptide); estrogens; erythropoietin (EPO); etc [23].

Cardioprotection in the diseased hearts

General remarks

In clinical situations, elderly patients or patients with heart diseases are at a high risk of Ischemia/reperfusion (I/R) injury. Some clinical and experimental studies provide evidence that aging or diseased myocardium is less amenable to protective measures.

Aged hearts:

With increasing age, there is a decrease in the ability of the myocardium to tolerate ischemic or hypoxic stress [59]. In 1996, Abete and colleagues reported that both single and multiple ischemic preconditioning can improve left ventricular function in adult (6 months old) rat hearts [60]. However, neither treatment can protect the senescent rat hearts (24 months old). Even worse, an increased deleterious effect of I/R injury was reported in preconditioned aged rat hearts [61]. In a clinical study, Abete and colleagues found that the presence of angina before acute myocardial infarction decreased the mortality in adults (< 65 years old) while not in elderly (> or = 65 years old) patients [62]. Another clinical study found that the beneficial effects (lower creatine kinase levels and better survival) associated with prodromal angina is lost in elderly patients with acute myocardial infarction [63]. Jiménez-Navarro M reported an opposite observation in which angina 1 week before a first infarction may protect against in-hospital adverse outcomes, improving left ventricular function and decreasing the incidence of arrhythmias [64].

Diabetes:

Diabetic hearts are more likely to develop vascular disease [65]. There are two types of diabetes: insulin-dependent diabetes (IDD) and non-insulin-independent diabetes (NIDD). In 1996, Tosaki A reported a loss of protection by IPC in streptozotocin-induced diabetic rat hearts [66]. In diabetic (3 weeks after streptozotocin-alloxan) dogs, treatment with diazoxide, a specific mK_{ATP} opener, was unable to decrease infarct size induced by I/R injury [67]. Another study found that isoflurane induced preconditioning was attenuated in diabetic dogs [68]. In a clinical study, Ghosh S found that patients with poor left ventricular intervention, IDD or NIDD, cannot be protected by IPC [69]. However, the application of diazoxide can mimic the protection in patients with poor left ventricular function except for patients with NIDD or IDD. In contrast, Liu et al. found that IPC decreases infarct size in NIDD rats (2 weeks after streptozotocin treatment) similarly as in normal hearts [70]. Delayed preconditioning was also lost in a sheep model of alloxan-induced diabetes [71].

Hyperlipidemia and atherosclerosis:

It is well known that there is a close relationship between the increase in serum total cholesterol concentration and the morbidity and mortality of myocardial infarction [65]. In an acute hypercholesterolemia rabbit model, coronary occlusion and reperfusion led to more extended myocardial infarction than in normal hearts [72]. Szilvassy reported for the first time that pacing-induced preconditioning is lost in a rabbit model of hypercholesterolemia and atherosclerotic where rabbits were treated for 8 weeks with high dietary cholesterol [73]. Interestingly, when these animals were reexposed to normal diet and when the serum lipid level recovered to normal level, the preconditioning treatment also regained its protective effect. In contrast, some studies showed an intact infarct size limiting effect of IPC in rabbits fed with a cholesterol-enriched diet [74], and in atherosclerotic ApoE/$LDLr^{-/-}$ double knockout mice fed with a cholesterol-enriched diet [75]. Delayed preconditioning [76,77] was also shown to be lost in rabbits with hypercholesterolemia. Iliodromitis EK and colleagues found in a recent research that IPostC is ineffective in limiting infarct size in rabbits with hypercholesterolemia and atherosclerosis [78].

Cardioprotection in remodelled hearts (see Fig.5)

Myocardial remodelling is an adaptive process to a variety of hemodynamic conditions associated with increased cardiac work. Short-term remodelling may result in a compensated cardiac function. Long-term remodelling may lead to heart failure. The remodelled myocardium changes in structure, morphology [79], metabolism [80], and cell signalling [81]. These changes put the remodelled myocardium at particular risk when exposed to ischemia. Hypertensive left ventricular hypertrophy and infarct induced remodelling are among the most common types of remodelling. The following paragraphs will discuss these two forms of remodelling and analyze the cardioprotective treatments in remodelled hearts.

Hypertensive left ventricular hypertrophy:

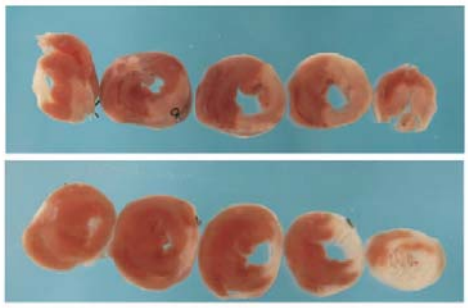
Hypertension is often underlying left ventricle hypertrophy. In 1994, IPC was found to decrease infarct size in adeoxycorticosteroid-treated and salt-fed (DOCA-salt) hypertensive rats to the same extent as observed in normotensive rats [82]. One year later, it was reported that IPC and adenosine can protect the spontaneously hypertensive rat heart [83], improving recovery of ATP, left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), $\pm$dP/dt, and coronary flow. The transgenic hypertensive rat heart can also be protected by IPC [84]. Aortic constriction modified hypertensive rat hearts were also shown to be protected by IPC [85]. However, IPC failed to protect patients with poor cardiac function [69]. In genetically hypertensive rat model, ischemic preconditioning was unable to recover the coronary flow similarly to normotensive rat hearts [86]. As for pharmacological preconditioning, Ebrahim Z found that bradykinin pre-treatment decreased infarct size less in DOCA-salt hypertensive rat hearts than in normotensive hearts [87].

Post infarct-remodelled myocardium:

A rabbit heart remodelling model can be induced induced by coronary ligation two weeks before experimentation. Using such a model, Miki T first found that IPC is less protective in decreasing infarct size in remodelled hearts than in sham operated hearts [88]. In follow-up studies, Miki T found that erythropoietin (EPO) can protect the remodelled hearts similarly to the sham hearts [89]. Pharmacological postconditioning induced by isoflurane can also be used to protect post-infarct remodelled rat hearts [90].

Fig.5

A.



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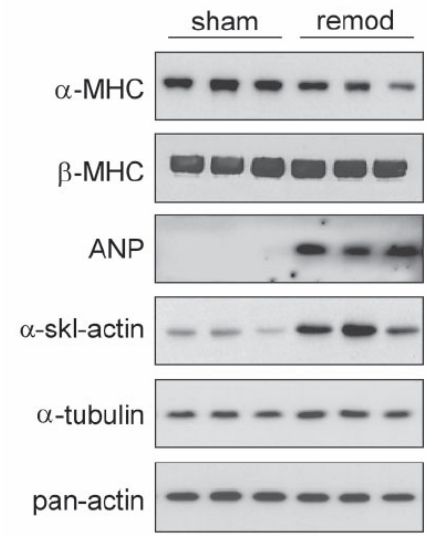


Fig.5 Cardiac remodelling: Left anterior descending coronary artery was ligated, and infarct size was determined after 12 h using 1% triphenyltetrazolium chloride staining (A, upper panel). Viable myocardium is stained brick-stone red, whereas freshly infarcted myocardium is stained salmon pink. Note the black ties on the two basal transverse heart sections. A mean infarct size of 35 ± 5% was obtained (see also table 1). Six weeks after ligation, necrosis was replaced by scar tissue, and compensatory hypertrophy developed (A, lower panel). Chronic infarct is stained bright white. Western blot analysis of remodeling markers (B): atrial natriuretic peptide (ANP), α- and β-myosin heavy chains (α- and β-MHC), α-skeletal-actin (α-sklactin). α-tubulin and total actin (pan-actin) were used as loading controls. Remod=postinfarct remodeled hearts; sham=sham-operated hearts. Data are given as mean±SD (n=5 in each group). *p < 0.05 versus sham. [Adopted from [90]]

Altered protection signalling in diseased hearts

General remarks

Aged and diseased hearts have shown special changes in cardioprotective signalling, which might account for the impairment or loss of cardioprotection by traditional pre- or postconditioning. Some of the altered protection signalling in these hearts will be discussed in the following paragraphs.

Aged hearts:

Aging is associated with increased oxidative stress, reduced mitochondrial membrane potential, and reduced ATP synthesis [65]. These changes may contribute to impaired energy reserve, impaired systolic and diastolic function and reduced tolerance to ischemic injury. The signalling and expression of cardioprotective proteins also change during aging, i.e., PKC [65], MAPK, heat shock protein 70 (HSP 70), NOS, and sodium-hydrogen exchanger (NHE) [59]. This may account for the decreased protection induced by IPC or pharmacological mimics. However, there are still treatments that successfully protected the aged heart. One effective intervention to protect aged hearts is to reduce energy demands during ischemia (for example during cardiac surgery) by β-adrenergic receptor blockers [91]. Interestingly, regular exercise can protect against I/R injury in both old and young animals [92], probably due to the increased expression level of HSPs, improve antioxidant capacity, and / or elevation of other carioprotective proteins.

Diabetes:

It was reported that during the early phase of experimentally induced diabetes (2 weeks) the diabetic heart is more resistant to ischemia/reperfusion [66]. However, the severely ill diabetic heart is more susceptible to ischemia/reperfusion. Studies indicate that PKC activation may be responsible for the increased resistance to ischemia in the early stage of diabetes [93]. Conversely, hyperglycemia is a risk factor, which contributes to the high susceptibility of diabetic hearts to ischemia/reperfusion. Hyperglycemia correlates with the motality after myocardial infarction in patients with or without diabetes [94-96]. Infarct size was also shown to correlate with the extent of hyperglycemia in dogs with or without diabetes [67,97]. Enhanced oxidative stress, accumulation of undesirable metabolites, and vascular dysfunction, may further contribute to high susceptibility of diabetic hearts to ischemia/reperfusion [93].

Activation of RISK pathways (Akt, ERK, PKC, etc) at the onset of reperfusion, mKATP channell opening, and preventation of mPTP opening are the critical events in cardiorotection induced by many treatments. Changes in all these events in the diabetic heart may interfere with protection by pre- and postconditioning.

High blood glucose levels can abolish cardioprotection induced by IPC [98], anesthetic-induced preconditioning [99], and delayed preconditioning [71]. A recent study showed that hyperglycemia can also abolish anesthetic-induced postconditioning [100]. One study showed that acute hyperglycemia leads to tyrosine nitration and apoptosis via increased iNOS gene expression and NO release in rat hearts [101]). Another study showed that hyperglycemia can lead to tyrosine nitration of PI3K, resulting in Akt inactivation and thus blocking the prosurvival effect of VEGF [102].

More cycles of preconditioning ischemia are required to gain similar activation of PI3K-Akt and protective effects in diabetic rat hearts than in normal hearts. This indicates that the impaired activation of PI3K-Akt pathway may be responsible for the loss of protection by IPC in diabetic hearts [103].

In a study with human cardiac tissue (atrial appendages) from patients with poor left ventricular intervention, IPC was not protective [69]. Interestingly, the application of diazoxide could restore the protection in patients with poor left ventricular function except in patients with non insulin dependent diabetes mellitus or insulin dependent diabetes mellitus, indicating that the failure to protect the heart by IPC is due to a dysfunction of the mK_{ATP} channel itself.

Besides the altered signalling in diabetic hearts, cardioprotection in diabetes is considered to be affected by anti-diabetes drugs. Some K_{ATP} channel inhibitors are used as anti-diabetic drugs because they increase insulin secretion by blocking these channels in the pancreatic β-cell membrane. Were these drugs not specific for pancreatic K_{ATP} channels (i.e. glibenclamide), they can also inhibit the mK_{ATP} channels in cardiomyocytes. This may increase the cardiovascular risk for diabetic patients and may also explain why diabetic patients are less protected by IPC. In contrast, the more selective pancreatic K_{ATP} channel inhibitors do not affect protection of preconditioning [104].

Treatment of diabetes with insulin activates the PI3K-Akt signalling pathway, one of the RISK pathways, and thus can avoid the blockade of K_{ATP} channels [65].

Hyperlipidemia and atherosclerosis:

The mechanisms by which hyperlipidemia affects the outcome of ischemia/reperfusion injury and cardioprotection induced by pre- and postconditioning is not very clear. Csont T and colleagues reported that there is a decrease in ischemia or heat shock induced HSP 70 expression in high cholesterol diet exposed rat hearts when compared to normal hearts [105]. Failure to induce tetrahydrobioperin synthesis (an important cofactor of critical enzymes including iNOS) by ischemic preconditioning was reported in a hyperlipidemia rabbit model, which may be responsible for the loss of late preconditioning [77]. Statins, the very commonly used antihyperlipidemic drugs, which inhibit biosynthesis of cholesterol, showed preconditioning mimicking effects [106].

Altered protection signalling in remodelled hearts

Hypertensive left ventricular hypertrophy:

There is considerable controversy whether hypertensive hearts can be protected by ischemic or pharmacological preconditioning. Also, few studies investigated the mechanisms underlying cardioprotection in hypertrophied hearts. In a study where NO donor was used to induce preconditioning, the spontaneously hypertensive rat (SHR) hearts were not protected. Further investigations found that NO induced cGMP expression in normotensive hearts but not in SHR hearts [107].

Post infarct-remodelled myocardium:

In a study where Miki T found that IPC is less protective in post-infarct remodelled rabbit hearts than in control hearts [88], chronic treatment of valsartan, an angiotensin II type 1 (AT1) receptor blocker, prevented remodelling and preserved the effect of IPC. This indicates that AT1 receptor activation is important for the loss of IPC in remodelled hearts. In the same study, application of diazoxide, the mK_{ATP} channel opener, protected both remodelled and healthy hearts, suggesting that the impaired mechanism in remodelled hearts is upstream of mK_{ATP} channel. In a follow up study by Miki T, PKC-ε activation in response to IPC was impaired in post-infarct

remodelled hearts [108]. In another study, PI3K/Akt activation by EPO receptor signalling was also shown to be impaired [89]. The same study further indicates the activation of an alternative protective signalling pathway by EPO in these hearts. One study by Feng J and colleagues (our own laboratory) showed that PI3K/Akt signalling pathway is critical in isoflurane-induced postconditioning in a post-infarct remodelled rat heart model [90].

C. Hypothesis and results

Part I: Ischemic postconditioning in hypertrophied myocardium and molecular mechanisms involved.

Hypothesis:

Remodelled hearts can be protected by ischemic postconditioning.

Two remodelled rat heart models were used in these experiments:

a. The myocardial infarction-induced cardiac hypertrophy and remodelling model
b. The Hypertension- and overload-induced cardiac hypertrophy model [1-kidney-1-clip (1K1C) hypertensive rat model]

Results:

1. **Cardioprotection by ischemic postconditioning is preserved in hypertrophied myocardium**

a. IPostC prevented myocardial damage in both MI-remodelled and 1K1C hearts, as measured by decreased infarct size and lactate dehydrogenase release, and improved function.
b. The maximal extent of infarct size reduction and left ventricular developed pressure (LVDP) achieved by IPostC were partially impaired in 1K1C hypertrophied hearts, but were unchanged in MI-remodelled hypertrophied hearts.
c. The maximal inotropic functional recovery in 1K1C hearts was not different from that of healthy hearts, but was less improved in MI hearts.
d. Inhibition of the phosphatidylinositol 3-kinase (PI3K) pathway with LY294002 abolished the protective effects of IPostC in both disease models and healthy hearts.

2. **Ischemic postconditioning activates PKB/Akt and its downstream targets GSK3β, eNOS, and p70S6K in a PI3K dependent manner in hypertrophied myocardium**

a. IPostC significantly increased phosphorylation of PKB/Akt and its downstream targets GSK3β, eNOS, and p70S6K in remodelled hearts.

b. IPostC-induced phosphorylation of PKB/Akt and its downstream substrates GSK3β, eNOS, and p70S6K were suppressed by LY294002.
c. The activity of PKB/Akt was significantly elevated by IPostC compared to the ischemic control and completely abolished by LY294002.
d. Phosphorylation and enzyme activity of PKB/Akt were partially increased by ischemia alone compared to the time-matched controls in both healthy and MI-remodelled hearts. However, this was not observed in the 1K1C hearts.

3. Cardioprotection by ischemic postconditioning in the remodelled myocardium does not primarily depend on ERK1/2 signaling

a. In healthy hearts, phosphorylation of ERK1/2 was moderately increased by ischemia alone, and was further enhanced by IPostC. In 1K1C hearts, phosphorylation of ERK1/2 was not increased by ischemia alone but was increased by IPostC. In contrast, phosphorylation of ERK1/2 in the MI-remodelled hearts was strongly increased by ischemia alone, but was not further increased by IPostC.
b. There was no increase in ERK1/2 activities by IPostC in both remodeling models as well as in healthy hearts.

Part II: Delayed pharmacological preconditioning in remodelled rat hearts.

Hypothesis:

Delayed pharmacological conditioning is preserved in post-infarct remodelled rat hearts.

Remodelled rat heart model:

The myocardial infarction-induced cardiac hypertrophy and remodelling model

Results:

1. Post-infarct remodelling narrows the second window of protection after isoflurane preconditioning.

a. Isoflurane induced delayed preconditioning in both post-infarct remodelled hearts and healthy hearts.
b. The second window of delayed preconditioning is narrower in remodelled hearts (24 h) than in healthy hearts (48 h).

2. Late protection by isoflurane preconditioning is more vulnerable to cyclooxygenase-2 inhibition in remodelled myocardium

a. Delayed protection by isoflurane is abolished or attenuated by inhibitors of COX-2 (celecoxib and NS-398), but not by 12-lipoxygenase (cinnamyl-3,4-dihydroxycyanocinnamate).
b. Isoflurane-induced late preconditioning is partially inhibited by celecoxib, a clinically used COX-2 inhibitor, in healthy myocardium. However, this late protection is completely abolished by celecoxib in remodelled myocardium.

3. Isoflurane-induced cyclooxygenase-2 expression and activity show alterations in remodelled hearts.

a. In healthy hearts, COX-2 expression was increased 24 (1.5-fold) and 48 h (2.7-fold) after isoflurane exposure, while in remodelled hearts, COX-2 expression was exclusively increased at 24 h, but not at 48 h.
b. Isoflurane-induced COX-2 showed a shortened activation profile in remodelled hearts, which closely paralleled structural and functional protection.

4. Isoflurane increased DNA binding activity of HIF1α but not CREB in post-infarct remodelled hearts.

a. DNA binding activity of HIF1α and CREB, but not NFκB or STAT3, increased at 30 min after isoflurane treatment in healthy hearts.
b. Isoflurane treatment only increased HIF1α DNA binding activity in remodelled hearts.
c. ICER, an antagonist of CREB, was significantly increased in infarct remodelled hearts.

D. Discussion

Part I

In this study we show for the first time that hypertrophied hearts can also be protected against I/R injury by IPostC (see Fig.6). Although IPostC was previously reported to be ineffective in limiting infarct size in rabbits with hypercholesterolemia and atherosclerosis [78], our results are in accordance with a recent clinical study [109], in which the human myocardium of patients undergoing primary percutaneous coronary intervention was protected by repetitively inflating and deflating an angioplasty balloon within minutes of reperfusion.

Fig.6

A. B.

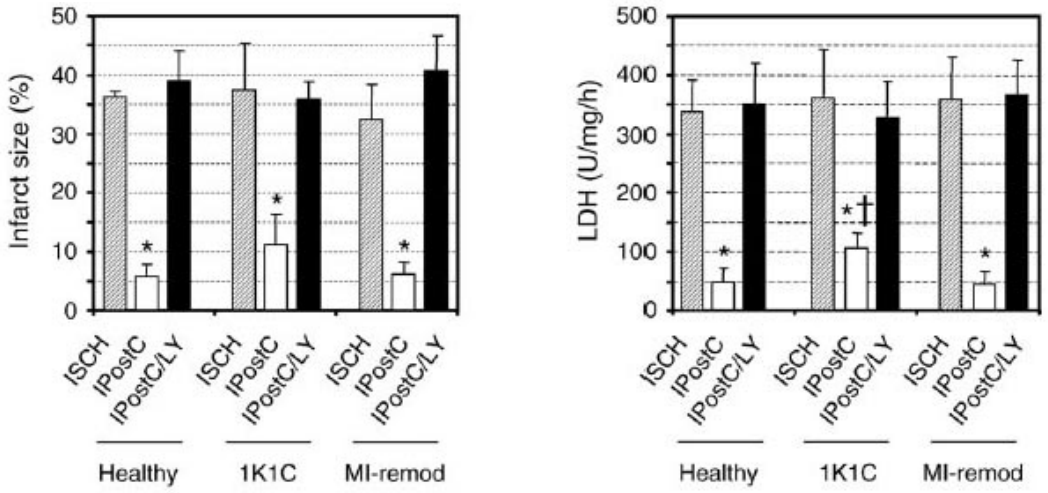


Fig.6 Infarct size (panel A), lactate dehydrogenase release (panel B) of healthy, 1K1C, and MI-remodeled hearts: ISCH: hearts exposed to 40 min of test ischemia followed by 90 min reperfusion. IPostC: ischemic postconditioning. IPostC/LY: postconditioned hearts exposed to the PI3K inhibitor LY294002 (15 µmol/l) during first 15 min of reperfusion. CTL: time-matched perfusion. Data are mean±S.D. (n=5 per group). *$p<0.05$ vs. ISCH and †$p<0.05$ vs. same treatment in healthy hearts.

Previous studies have shown that the PI3K-PKB/Akt and ERK1/2 signalling pathways are the major survival kinase pathways involved in ischemic preconditioning and

postconditioning [13]. However, the relative importance of the two kinase pathways in mediating the protection is not yet clear. A study investigating pharmacological postconditioning by 5′-(N-ethylcarboxamido) adenosine and bradykinin in isolated rabbit hearts suggests that PKB/Akt is upstream of ERK1/2 [110], while a more recent study in isolated perfused rabbit hearts stresses the pivotal role of ERK1/2 instead of PI3K–PKB/Akt in IPostC-mediated protection [44]. Another study reported that IPostC failed to protect against ischemic injury in pigs though both PKB/Akt and ERK1/2 are activated[111]. Here we show that PKB/Akt is markedly activated by IPostC in two hypertrophied heart models as well as in healthy hearts (see Fig.7). We also show that the phosphorylation levels of GSK3β, eNOS, and p70S6K, the downstream targets of PI3K-PKB/Akt pathway, are elevated by IPostC. Activation of PKB/Akt and phosphorylation of its downstream targets are tightly linked with functional and structural protection and are sensitive to inhibition by LY294002. Our results also provide evidence that the PKB/Akt pathway is critical in IPostC induced cardioprotection in hypertrophied heart models. In our experiments, we observed an increase in ERK1/2 phosphorylation by IPostC in healthy and 1K1C hearts. However, the catalytic activity of ERK1/2 was not elevated by IPostC in healthy and diseased hearts. These data suggest that PI3K–PKB/Akt but not ERK1/2 is the predominant mediator of IPostC-induced protection in both healthy and diseased hearts.

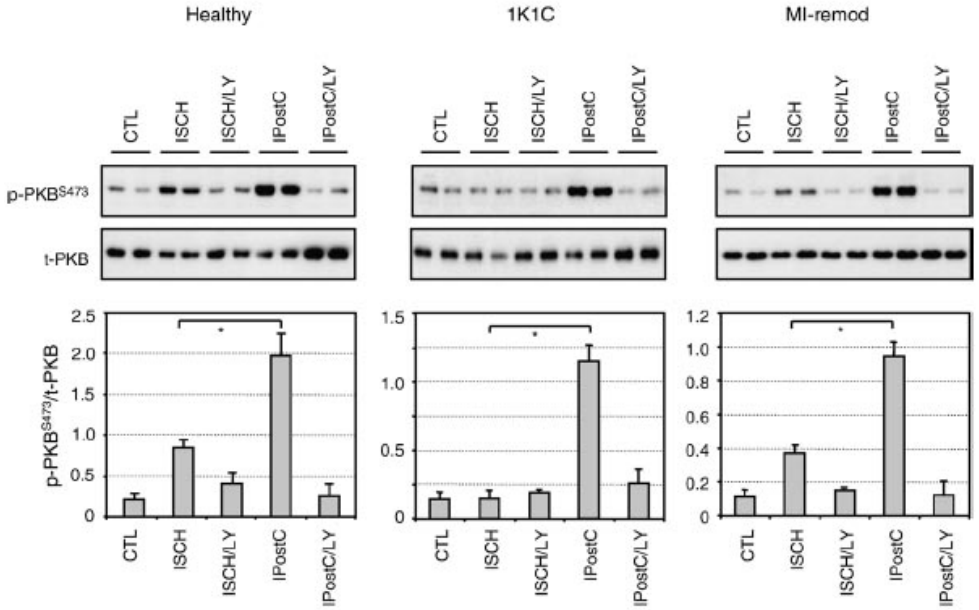
We found some noteworthy differences in PKB and ERK1/2 signaling in different disease models. First, although the maximal activation of PKB/Akt can be achieved by IPostC in all three models, partial activation of PKB/Akt by ischemia alone was impaired in 1K1C hearts. This may explain why the maximal extent of infarct size reduction and the recovery of left ventricular developed pressure (LVDP) achieved by IPostC were partially impaired in 1K1C rat hearts. Second, the basal kinase activity of ERK1/2 in MI-remodelled hearts was different from that observed in healthy hearts. These suggest that the key players of the RISK pathways may be differentially regulated by different types of cardiac remodelling. Finally, the recovery of inotropy achieved by IPostC was impaired in MI-remodelled hearts. The reason for these differences is unclear.

In summary, we show in our study that remodelled rat hearts with hypertrophy induced by infarction (permanent ligation of coronary artery) and 1K1C hypertension are still receptive to protection by IPostC. Furthermore, we identified in these models

the PI3K–PKB/Akt signalling pathway as predominant mediator of IPostC-induced cardioprotection.

Fig.7

A.



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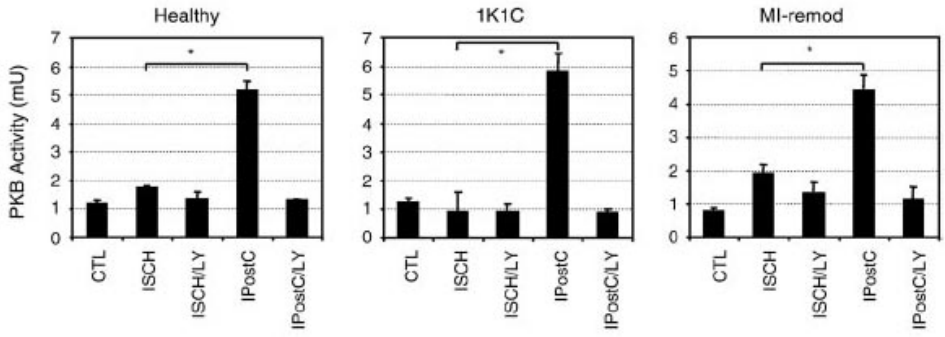


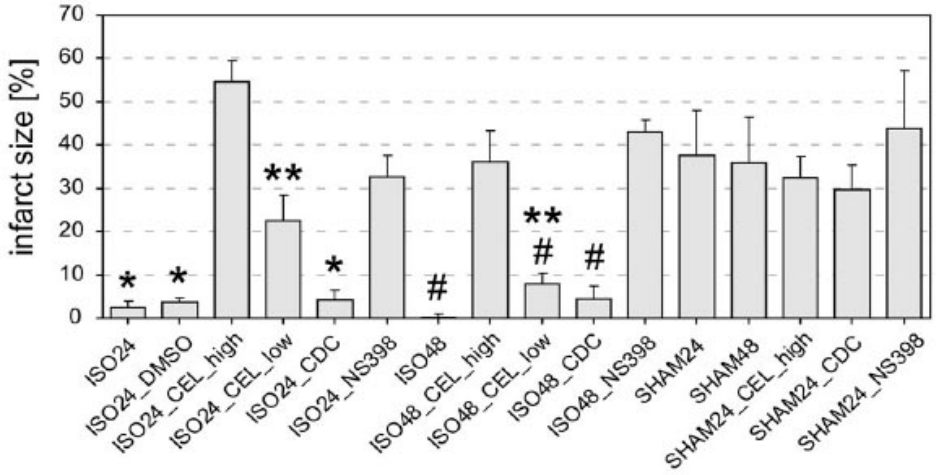
Fig.7 Phosphorylation status (panel A) and kinase activity (panel B) of protein kinase B/Akt (PKB): Representative Western blots and average density ratio of p-PKB^{S473}/total-PKB for each group. Data are mean±S.D. (n=4 per group). *$p<0.05$ vs. ISCH.

Part II

In this study we found for the first time that isoflurane-induced late preconditioning is preserved in post infarct-remodelled rat hearts. However, the window of late preconditioning is shorter in the remodelled hearts: reduction in infarct size and improvement of functional recovery were observed only at 24 hours after isoflurane treatment, while in healthy hearts protection was still observed 48 hours after isoflurane treatment (see Fig.8).

Fig.8

A. Healthy



B. Remodelled

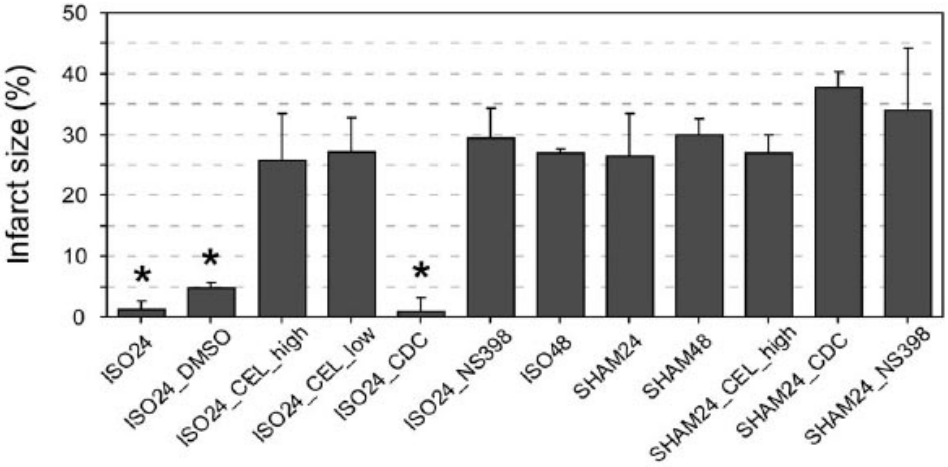


Fig.8 Infarct size in healthy (A) and remodelled (B) hearts: ISO24/48: hearts exposed to ischaemia-reperfusion 24 or 48 h after isoflurane preconditioning.

ISO24/48_CEL_high: preconditioned hearts treated with 1 μM celecoxib. ISO24/48_CEL_low: preconditioned hearts treated with 0.1 μM celecoxib. ISO24/48_CDC: preconditioned hearts treated with 0.5 μM cinnamyl 3,4-dihydroxy-cyanocinnamate (CDC). ISO24/48_NS398: preconditioned hearts treated with 5 μM N-2-cyclohexyloxy-4-nitrophenyl-methane-sulphonamide (NS398). Final concentration of dimethyl sulphoxide (DMSO) was < 0.1%. The prefix SHAM in group names indicates respective groups without isoflurane preconditioning (oxygen alone). *$p < 0.05$ vs. SHAM24 groups. #$p < 0.05$ vs. SHAM48 groups. **$p < 0.05$ vs. high celecoxib concentration. Data are mean±SD (n=5 per group).

Isoflurane-induced delayed preconditioning was mediated by COX-2 but not 12-lipoxygenase in both remodelled and healthy rat hearts. This is consistant with a previous report in which isoflurane-induced delayed preconditioning in healthy rabbits was inhibited by the COX-2 inhibitor celecoxib [112]. We further found that the protection in both remodelled and healthy hearts was closely correlated with increased COX-2 protein level and activity. While isoflurane increased DNA binding activity of COX-2-inducing HIF-1α in healthy and remodelled hearts, it failed to increase DNA binding activity of CREB (also a COX-2-inducing transcription factor) in post-infarct remodelled hearts. This may account for the impaired expression and activation of COX-2 and thus the narrowing of the second window of protection in remodelled hearts. Furthermore, despite increased expression of COX-2, remodelled preconditioned hearts were much more vulnerable to the clinically used anti-inflammatory drug celecoxib than healthy hearts (see Fig.9). This finding may be of clinical importance that patients at risk of cardiovascular complications should avoid using nonsteroidal anti-inflammatory medication.

Compared to the early window of preconditioning, delayed (or the second window of) preconditioning has strong and more reliable anti-stunning and anti-infarct effects, and lasts for a longer time, making it more clinically relevant. Delayed preconditioning causes an increase of prostonoids in cardiac tissue via enhanced expression of COX-2, the rate limiting enzyme in the synthesis of prostaglandin [19], converting arachidonic acid to prostonoids. COX-2 is usually expressed at low levels in the heart [113] but can be rapidly induced by activation of multiple transcription factors.

Prostanoids can open mK_{ATP} channels, the key players in cardioprotection [114], leading to cardioprotection. Inhibition of COX-2 abolishes the increase in tissue levels of prostanoids, anti-stunning effects, and infarct limiting effects of delayed preconditioning [115]. Previous studies using healthy animal models of different species reported volatile anesthetics induced late preconditioning [116,117]. Studies on the mechanisms of anesthetics-incuded delayed preconditioning identified reactive oxygen and nitrogen species as triggers [118], inducible nitric oxide synthase [119], endothelial nitric oxide synthase [120], and 12-lipoxygenase [121] as mediators, and sarcolemmal and mitochondrial K_{ATP} channels as end effectors [122]. However, till now, only one study with healthy rabbit hearts indicated the role of COX-2 in isoflurane-induced delayed preconditioning using the clinically used COX-2 inhibitor celecoxib [112]. Our present study confirms COX-2 as a critical mediator in isoflurane-induced delayed preconditioning in both healthy rat hearts and post-infarct remodelled myocardium.

Fig.9

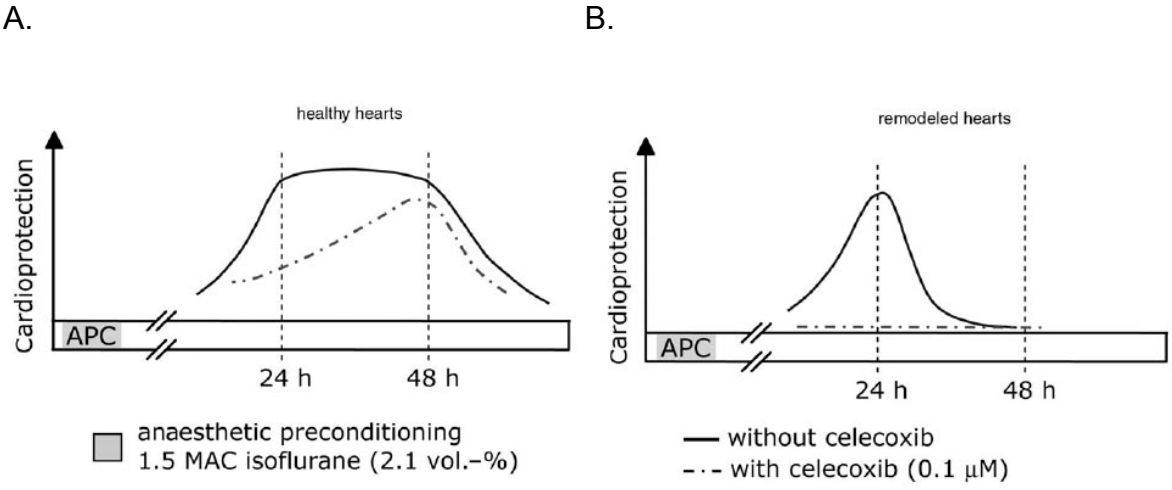


Fig.9 Second window of protection in healthy and remodelled hearts: The panels show the second window of protection at 24 and 48 h after isoflurane exposure (APC) in healthy (A) and infarct remodelled (B) hearts in the absence (solid line) and presence (dashed line) of celecoxib (0.1 μM).

The remodelled myocardium is at a high risk for further ischemic damage due to changes in structure [79], metabolism [80], and cellular signalling [123]. Ischemic

preconditioning is reported to be less protective in the remodelled rabbit myocardium than in sham operated hearts [88]. Several studies reported altered cardioprotective signalling in remodelled hearts [89,108]. However, no data is available with respect to delayed pharmacologic or ischemic preconditioning in post-infarct remodelled hearts. Our previous studies on early classic isoflurane-induced preconditioning [124] and postconditioning in remodelled myocardium [90] found that protection against ischemia-reperfusion injury was fully preserved. However, our present study indicates that ventricular remodelling makes the heart more vulnerable to ischemia-reperfusion injury. To study the mechanisms underlying this vulnerability, we tested a number of COX-2-inducing transcription factors to see whether their DNA-binding activity would be impaired in the diseased state. We observed increased DNA-binding activity of HIF 1α in both healthy and diseased hearts after isoflurane treatment. This is consistant with a previous report that isoflurane can increase protein levels of HIF 1α in rat myocardium [125]. In contrast, the DNA binding activity of CREB was increased by isoflurane in healthy but not in remodelled hearts. This might be due to the increased expression of ICER, the transcriptional repressor and CREB-antagonist [126], in remodelled hearts. However, our experiments can not exclude that other additional mechanisms contributed to the shorter duration of COX-2 upregulation in remodelled hearts.

COX-2 inhibitors increase the number of cardiovascular complications specifically in patients with preexisting heart disease [127]. A recent study in pig showed that peri-infarct inhibition of COX-2 by celecoxib decreased myocardial function and increased left ventricular remodeling and mortality [128]. In the current study, we also evaluated the effects of celecoxib on isoflurane-induced delayed cardiac protection. Our results showed that low concentrations of celecoxib are sufficiant to abolish delayed protection induced by isoflurane. From a translational point of view, our data further indicate that the use of COX-2 inhibitors should be minimized in patients with significant ventricular remodelling.

Finally, the narrow window of isoflurane induced delayed preconditioning and the short period of elevated COX-2 expression imply that diseased myocardium requires more frequent intermittent preconditioning stimuli to maintain the protected state.

In summary, our study shows that remodelling in rat hearts impedes sustained expression and activation of COX-2 after isoflurane preconditioning, narrowing the second window of cardioprotection. Isoflurane increases DNA binding activity of HIF1α in both healthy and remodelled hearts, but fails to increase DNA binding activity of CREB in remodelled, ICER-overexpressing hearts. Our study further demonstrates that protection of remodelled myocardium is exceptionally vulnerable to COX-2 inhibitors. Thus, isoflurane-induced delayed cardioprotection varies with the disease state of the heart and concomitant medication.

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remodeling, and impairs systolic function after myocardial infarction in the pig. Circulation 2007; 115: 326-32

Contribution of Min Zhu to the following publications

1. **Zhu M,** Feng J, Lucchinetti E, Fischer G, Xu L, Pedrazzini T, Schaub MC, Zaugg M. Ischemic postconditioning protects remodeled myocardium via the PI3K-PKB/Akt reperfusion injury salvage kinase pathway. Cardiovasc Res. 2006;72:152-162.

- **Contribution: Study design and planning of experiments, preparation of samples, verification of remodelling via qRT-PCR of markers such as ANP, BNP etc, Gomori staining of tissue slices form hypertrophied or healthy hearts, measurement of protein phosphorylation via Western blot analysis. Analysis of data and interpretation.**

2. Feng J, Lucchinetti E, Fischer G, **Zhu M**, Zaugg K, Schaub MC, Zaugg M. Cardiac remodeling hinders activation of cyclooxygenase-2, diminishing protection by delayed pharmacologic preconditioning: Role of HIF1α and CREB. Cardiovasc Res. 2008;78:98-107.

- **Contribution: Study design and planning of experiments, sample preparation, measurement of protein level via Western blot, and measurement of binding activity of transcription factors via electrophoretic mobility shift assay. Analysis of data and interpretation.**

3. Lucchinetti E, Aguirre J, Feng J, **Zhu M**, Suter M, Spahn DR, Härter L, Zaugg M. Molecular evidence of late preconditioning after sevoflurane inhalation in healthy volunteers. Anesth Analg. 2007;105:629-640

- **Contribution: RNA extraction, qRT-PCR.**

4. Lucchinetti E, Hofer C, Bestmann L, Hersberger M, Feng J, **Zhu M**, Furrer L, Schaub MC, Tavakoli R, Genoni M, Zollinger A, Zaugg M. Gene regulatory control of myocardial energy metabolism predicts postoperative cardiac function in patients undergoing off-pump coronary artery bypass graft surgery: inhalational versus intravenous anesthetics. Anesthesiology. 2007;106:444-457.

- **Contribution: RNA extraction, synthesis of cDNA, qRT-PCR, gene chip analysis together with Dr. Eliana Lucchinetti.**

5. Feng J, Fischer G, Lucchinetti E, **Zhu M**, Bestmann L, Jegger D, Arras M, Pasch T, Perriard JC, Schaub MC, Zaugg M. Infarct-remodeled myocardium is receptive to protection by isoflurane postconditioning: role of protein kinase B/Akt signaling. Anesthesiology. 2006;104:1004-1014.

- **Contribution: Study design and planning of experiments, preparation of samples; verification of remodelling via qRT-PCR of markers such as ANP, BNP etc; immunofluorescence; measurement of protein phosphorylation via Western blot analysis. Analysis of data and interpretation.**

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Ischemic postconditioning protects remodeled myocardium via the PI3K–PKB/Akt reperfusion injury salvage kinase pathway

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Abstract

Objective: We tested whether ischemic postconditioning (IPostC) is protective in remodeled myocardium.
Methods: Post-myocardial infarct (MI)-remodeled hearts after permanent coronary artery ligation and one kidney one clip (1K1C) hypertensive hearts of male Wistar rats were exposed to 40 min of ischemia followed by 90 min of reperfusion. IPostC was induced by six cycles of 10 s reperfusion interspersed by 10 s of no-flow ischemia. Activation of reperfusion injury salvage kinases was measured using Western blotting and *in vitro* kinase activity assays.
Results: IPostC prevented myocardial damage in both MI-remodeled and 1K1C hearts, as measured by decreased infarct size and lactate dehydrogenase release, and improved function. The reduction in infarct size and the recovery of left ventricular contractility achieved by IPostC was less in 1K1C hearts, but was unchanged in MI-remodeled hearts when compared to healthy hearts. In contrast, the recovery of inotropy was unaffected in 1K1C hearts, but was less in MI-remodeled hearts. Inhibition of the phosphatidylinositol 3-kinase (PI3K) pathway with LY294002 abolished the protective effects of IPostC on both disease models and healthy hearts. Western blot analysis in conjunction with *in vitro* kinase activity assays identified protein kinase B (PKB)/Akt but not p42/p44 extracellular-signal regulated kinase 1/2 (ERK1/2) as the predominant kinase in IPostC-mediated cardioprotection in remodeled hearts. IPostC increased phosphorylation of the PKB/Akt downstream targets eNOS, GSK3β, and p70S6K in remodeled hearts.
Conclusion: Our results offer evidence that IPostC mediates cardioprotection in the remodeled rat myocardium primarily via activation of the PI3K–PKB/Akt reperfusion injury salvage kinase pathway.

Keywords: Cardiac remodeling; Ischemia–reperfusion injury; Postconditioning; Reperfusion injury salvage kinase; Cellular signaling

1. Introduction

Despite the powerful protective effects of preconditioning, the clinical application of this phenomenon has been rather disappointing, mainly because preconditioning must be instituted before the ischemic event [1]. In contrast, a more promising approach to cardioprotection termed "ischemic postconditioning" (IPostC) has been described by Vinten–Johansen's group [2]. It consists of several cycles of coronary occlusion/reperfusion started immediately at the

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onset of restoration of the coronary flow after prolonged ischemia. Unlike preconditioning, postconditioning theoretically allows unrestricted application in the clinical settings, and thus has attracted much attention over the past years. Importantly, some pharmacological agents can afford comparable protection when applied during early reperfusion ("pharmacological postconditioning") [3–5].

Recent studies revealed that IPostC exerts its protective effects through the recruitment of prosurvival kinases such as phosphatidylinositol 3-kinase (PI3K)–protein kinase B (PKB)/Akt and the p42/p44 extracellular signal-regulated kinase 1/2 (ERK1/2) pathways (also termed reperfusion injury salvage kinase or RISK pathway) at the time of reperfusion [6]. So far, nearly all experimental studies have evaluated IPostC in healthy juvenile hearts. However, this is far from clinical reality, where a high number of elderly patients with cardiovascular disease would benefit most from protection by IPostC. Hypertensive left ventricular hypertrophy and post-myocardial infarct (MI)-remodeled hypertrophy account for most of the clinically relevant cases of cardiac hypertrophy and remodeling. These hypertrophied and remodeled hearts, even during the compensated state, are at greater risk to suffer severe ischemic damage and may be less amenable to protection by postconditioning [7]. We therefore tested whether protection by IPostC would be diminished or lost in two highly controlled experimental models of markedly remodeled hypertrophic hearts.

The data provided in this study show for the first time that cardioprotection by IPostC is preserved in the remodeled myocardium after permanent coronary artery ligation and in one kidney one clip (1K1C) hypertensive rat hearts. Using *in vitro* kinase assays to directly measure PKB/Akt and ERK1/2 activities, we further identified PI3K–PKB/Akt as the predominant RISK pathway for IPostC-induced protection in both disease models.

2. Methods

This study was conducted in accordance with the guidelines of the Animal Care and Use Committee of the University of Zurich, Zurich, Switzerland. All experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 85-23, revised 1996).

2.1. Cardiac hypertrophy models

Myocardial infarction (~35% of left ventricular mass) and subsequent hypertrophic remodeling was induced in male adult (180–200 g, 8–9 weeks old) Wistar rats by permanent ligation of the left anterior descending coronary artery (LAD) under anesthesia, as previously described in detail [8,9]. Sham-operated animals underwent the same procedure except that the suture was passed under the coronary artery without ligation. To induce hypertensive cardiac hypertrophy, rats were subjected to right nephrectomy and a silver clip (0.2 mm passage) was placed around the left renal artery under anesthesia (1K1C) [10]. Control sham rats (1K) had right nephrectomy without application of a clip. Rats of both disease models were sacrificed 6 weeks after surgery, and the body weight and heart weight were measured. Systolic blood pressure was measured in awake rats by means of the tail-cuff method.

2.2. Histology

Left ventricular tissue samples were fixed with 3.6% formaldehyde and embedded in paraffin. 3-μm sections at mid-ventricular level were subjected to Gomori's silver staining and nuclear fast red for visualization of individual myocytes in the viable left ventricular wall. The stained sections were analyzed by microscopy using image analysis (Zeiss KS 400, Germany).

2.3. Experimental protocol of isolated perfused rat hearts in the Langendorff apparatus

Six weeks after surgery, rats were heparinized (500 units i.p.) and 15 min later decapitated. Hearts were removed and perfused in a non-circulating Langendorff apparatus with Krebs–Henseleit buffer gassed with 95% O_2 and 5% CO_2 at pH 7.4 and 37 °C [4]. Experimental conditions including coronary flow and temperature were carefully monitored throughout the protocols. After equilibration, perfusion pressure was set at 80 mm Hg and left ventricular end-diastolic pressure (LVEDP) at 10 mm Hg. Spontaneously beating hearts were exposed to 40 min of global no-flow ischemia (test ischemia) followed by 90 min of reperfusion (Fig. 1). IPostC was induced by six cycles of 10 s reperfusion interspersed by 10 s no-flow ischemia immediately after test ischemia. Five hearts were assigned to each of the following six groups: (1) CTL, time-matched perfusion without ischemia; (2) ISCH, test ischemia followed by 90 min reperfusion; (3) ISCH/LY, application of the PI3K inhibitor LY294002 (15 μmol/l) during the first 15 min of reperfusion; (4) IPostC, immediately applied after prolonged test ischemia; (5) IPostC/LY, application of LY for 15 min starting immediately at reperfusion in postconditioned hearts; and (6) vehicle, application of 0.02% dimethyl sulfoxide (DMSO) vehicle without LY294002 during 15 min of reperfusion. The following parameters were recorded: left ventricular developed pressure (LVDP) and derivatives ($\pm dp/dt$), left ventricular end-diastolic pressure (LVEDP), epicardial ECG, coronary flow (CF), and perfusion pressure. Infarct size was determined by 1% 2,3,5-triphenyltetrazolium chloride staining after 90 min of reperfusion [4]. In addition, myocardial damage was estimated by measuring the release of lactate dehydrogenase (LDH) from necrotic tissue, as previously described [8]. Briefly, the perfusate was collected and LDH activity was determined by the Roche/Hitachi 917 kit (sensitivity 6 U/l, intra- and interassay coefficients of variance < 1%). For Western blot analysis and

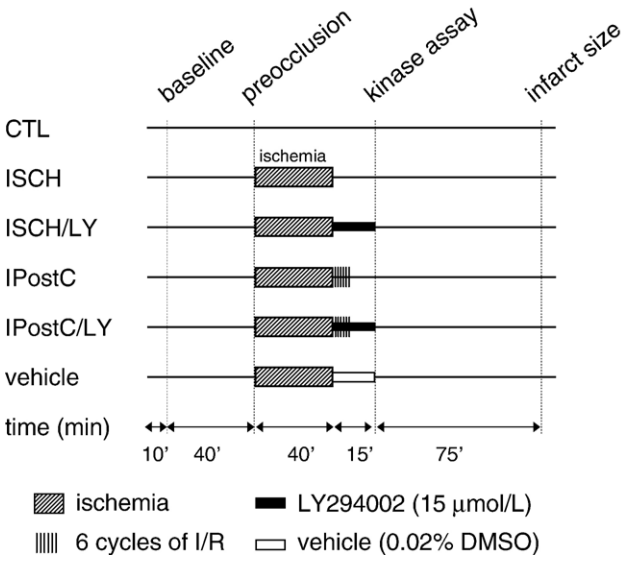

Fig. 1. Treatment protocols. ISCH: hearts exposed to 40 min of test ischemia followed by 90 min reperfusion. IPostC: ischemic postconditioning was induced by 6 cycles of 10 s reperfusion followed by 10 s no-flow ischemia right at the onset of reperfusion after 40 min of test ischemia. IPostC/LY: postconditioned hearts exposed to the PI3K inhibitor LY294002 (15 µmol/l) dissolved in vehicle (0.02% dimethyl sulfoxide (DMSO)) during the first 15 min of reperfusion. CTL: time-matched perfusion. Infarct size was determined by 1% triphenyltetrazolium staining at the end of experiments ($n=5$ hearts per experimental group). In separate experiments ($n=4$ hearts per experimental group), phosphorylation (by Western blotting) and activity of PKB/Akt and extracellular-signal regulated kinase 1/2 (ERK1/2) were assayed after 15 min of reperfusion.

in vitro kinase assays, separate experiments ($n=4$ in each group) were carried out and terminated after 15 min of reperfusion.

2.4. mRNA extraction and real-time quantitative polymerase chain reaction

Total RNA was prepared using RNeasy Mini Kit (Qiagen). First strand cDNA was synthesized from 1.0 µg of total RNA using SuperScript II reverse transcriptase (Invitrogen) and oligo-dT as primer. Real-time quantitative PCR was performed as previously described [8] using the following primers: atrial natriuretic peptide (ANP), 5′–ATCAC CAAGGGCTTCTTCCT–3′ (sense) and 5′–TGTTGGA CACCGCACTGTAT–3′ (antisense); and α-skeletal actin (α-skl-actin), 5′–CACGGCATTATCACCAACTG–3′ (sense) and 5′–CCGGAGGCATAGAGAGACAG–3′ (antisense). α-Tubulin was used for normalization with the sense and antisense primers 5′–CCATGCGTGAGTGTATCTCC–3′ and 5′–GTGCCAGTGCGAACTTCATC–3′, respectively.

2.5. Western blot analysis

Western blot analysis was carried out as previously described [8]. The antibodies used were from the following sources: glycogen synthase kinase 3β (GSK3β), p70S6K, endothelial nitric oxide synthase (eNOS), ERK1/2, phospho-PKB/Akt (Ser473), phospho-GSK3β (Ser9), phospho-p70S6K (Thr389), phospho-eNOS (Ser1177), phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology); monoclonal antibodies against pan-actin (Chemicon) and α-tubulin (Sigma); polyclonal antibody against ANP (Santa Cruz); polyclonal antibody against α-skl-actin was a gift from Dr. Christine Chaponnier (Department of Pathology, University of Geneva, Geneva, Switzerland); polyclonal anti-PKB/Akt antibody (Ab10) was a gift from Dr. Brian A. Hemmings (Friedrich Miescher Institute, Basel, Switzerland).

2.6. Immunoprecipitation and in vitro kinase assay

Left ventricular non-infarcted tissue (transmural) was extracted in ice-cold lysis buffer (25 mmol/l Tris–HCl, pH 7.4, 120 mmol/l NaCl, 2 mmol/l ethylenediamine tetraacetic acid, 1% Triton X-100, 0.5% Nonidet P-40, 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l benzamidine, 0.1 mmol/l sodium orthovanadate, 10 mmol/l sodium fluoride, and 1 µmol/l microcystin-LR). Extracts were centrifuged (16 000×*g*, 30 min, 4 °C), and protein concentrations determined using the Bradford assay. Tissue extracts (1 mg protein) were incubated at 4 °C for 2 h on a shaking

Table 1
Heart weight, body weight, and baseline cardiac function in the hypertrophic rat models

Rat model	Healthy	1K1C model		Infarct model	
		Sham (1K)	1K1C	Sham	MI-remod
HW (g)	1.203±0.089	1.204±0.070	1.927±0.191*	1.150±0.104	1.598±0.083*
BW (kg)	0.309±0.018	0.291±0.022	0.289±0.017	0.302±0.020	0.296±0.020
HW/BW (g/kg)	3.900±0.280	4.172±0.442	6.684±0.695*	3.817±0.401	5.428±0.413*
LVDP (mm Hg)	93.9±6.3	91.8±5.3	112.5±10.4*	91±4.0	67.7±5.6*
CF (ml/min)	13.1±1.2	12.9±1.1	13.4±1.3	13.1±3.2	13.4±1.3
HR (beats/min)	290±22	294±17	293±17	293±14	295±17

HW: heart weight; BW: body weight; LVDP: left ventricular developed pressure; CF: coronary flow; HR: heart rate; healthy: age-matched rats (300 g) serving as healthy controls. 1K1C: one kidney one clip rat model; sham (1K), nephrectomy without applying a clip (one kidney); MI-remod: postinfarct-remodeled rat hearts (permanent coronary artery ligation); sham: sham-operated rats (a suture was passed under the coronary artery without ligation). Rats in the hypertrophic model groups were used 6 weeks after surgery. Data are mean±S.D. ($n=20$ for sham (1K) and sham, $n=30$ for healthy, 1K1C, and MI-remod).

* $p<0.001$ vs. sham and healthy groups.

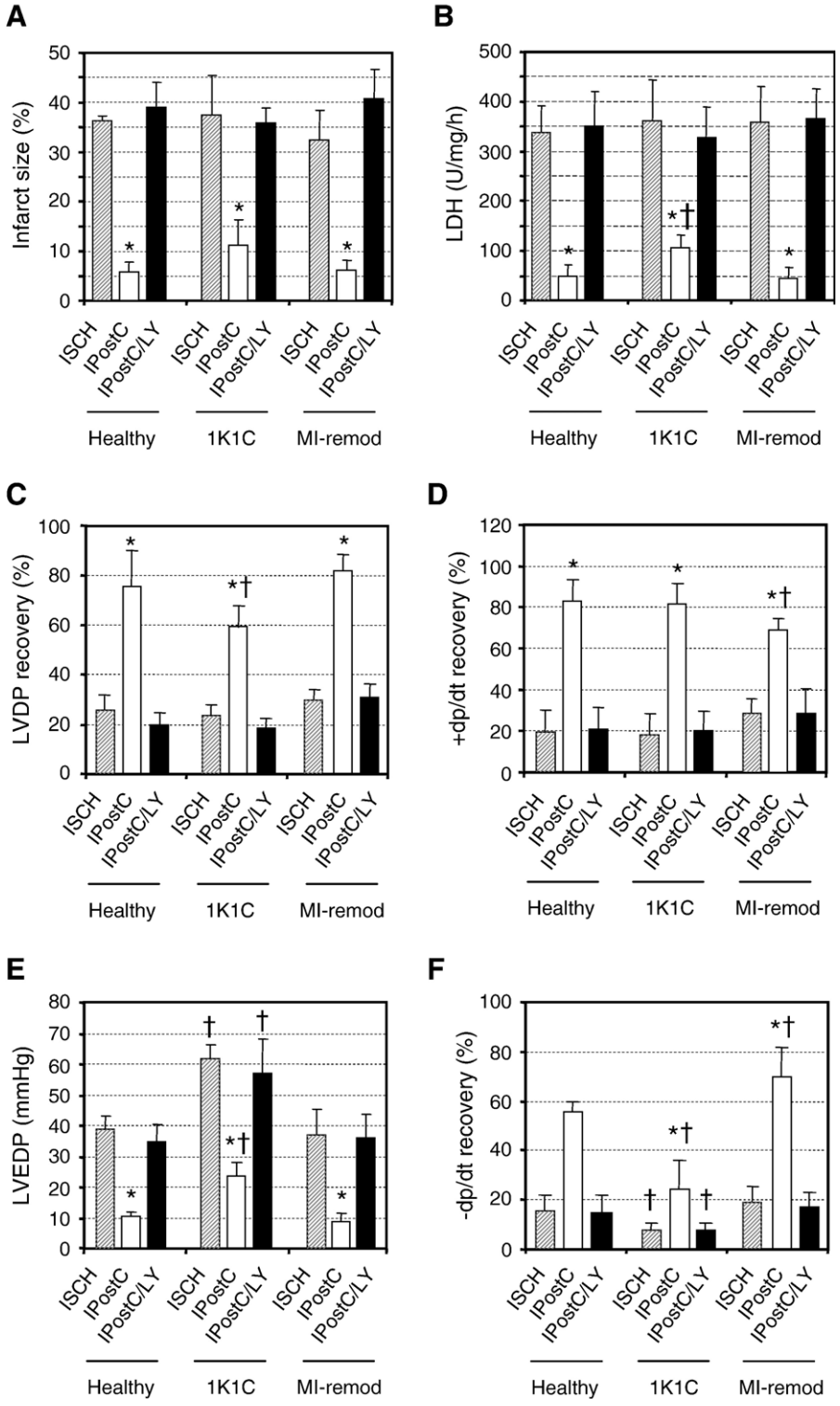

Fig. 2. Infarct size (panel A), lactate dehydrogenase release (panel B) and contractility parameters (panels C–F) of healthy, 1K1C, and MI-remodeled hearts. Left ventricular developed pressure (LVDP) (panel C), $+dp/dt$ (panel D), left ventricular end-diastolic pressure (LVEDP) (panel E), and $-dp/dt$ (panel F) were determined at 90 min of reperfusion and are indicated as percentage of baseline or mm Hg (LVEDP). ISCH: hearts exposed to 40 min of test ischemia followed by 90 min reperfusion. IPostC: ischemic postconditioning. IPostC/LY: postconditioned hearts exposed to the PI3K inhibitor LY294002 (15 μmol/l) during first 15 min of reperfusion. CTL: time-matched perfusion. Data are mean±S.D. ($n=5$ per group). *$p<0.05$ vs. ISCH and $^{\dagger}p<0.05$ vs. same treatment in healthy hearts.

platform with 2 μg of either polyclonal anti-PKB (Ab10) antibody coupled to 10 μl protein A-sepharose (Amersham Biosciences) or monoclonal anti-ERK1/2 (Zymed, clone ERK-7D8) coupled to 10 μl protein G-sepharose (Amersham Biosciences). The immune-complexes were then washed 4 times with lysis buffer, once with kinase buffer (25 mmol/l Tris–HCl, pH 7.4, 10 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol, 1 μmol/l PKI, and 1 μmol/l microcystin-LR), and finally resuspended in 20 μl of kinase buffer. For PKB kinase assay, the reaction was started with 60 μmol/l of Crosstide (GRPRTSSFAEG) and 20 μmol/l of [γ-^{32}P] ATP (Amersham Biosciences) in a final volume of 30 μl, incubated at 30 °C for 60 min, and stopped by addition of trichloroacetic acid (TCA) to a final concentration of 10%. After brief centrifugation, 20 μl aliquots were removed and spotted onto P81 phosphocellulose paper. The paper was washed immediately for 10 min with 75 mmol/l phosphoric acid (4 times), dried, and ^{32}P incorporation determined by Cerenkov counting. For ERK1/2 kinase assay, the reaction was started with 4 μg of myelin basic protein and 20 μmol/l of [γ-^{32}P] ATP in a final volume of 30 μl and incubated at 30 °C for 30 min. The reaction was terminated by addition of SDS-sample buffer and boiled at 95 °C for 5 min. Aliquots were subjected to SDS–PAGE (15%) followed by

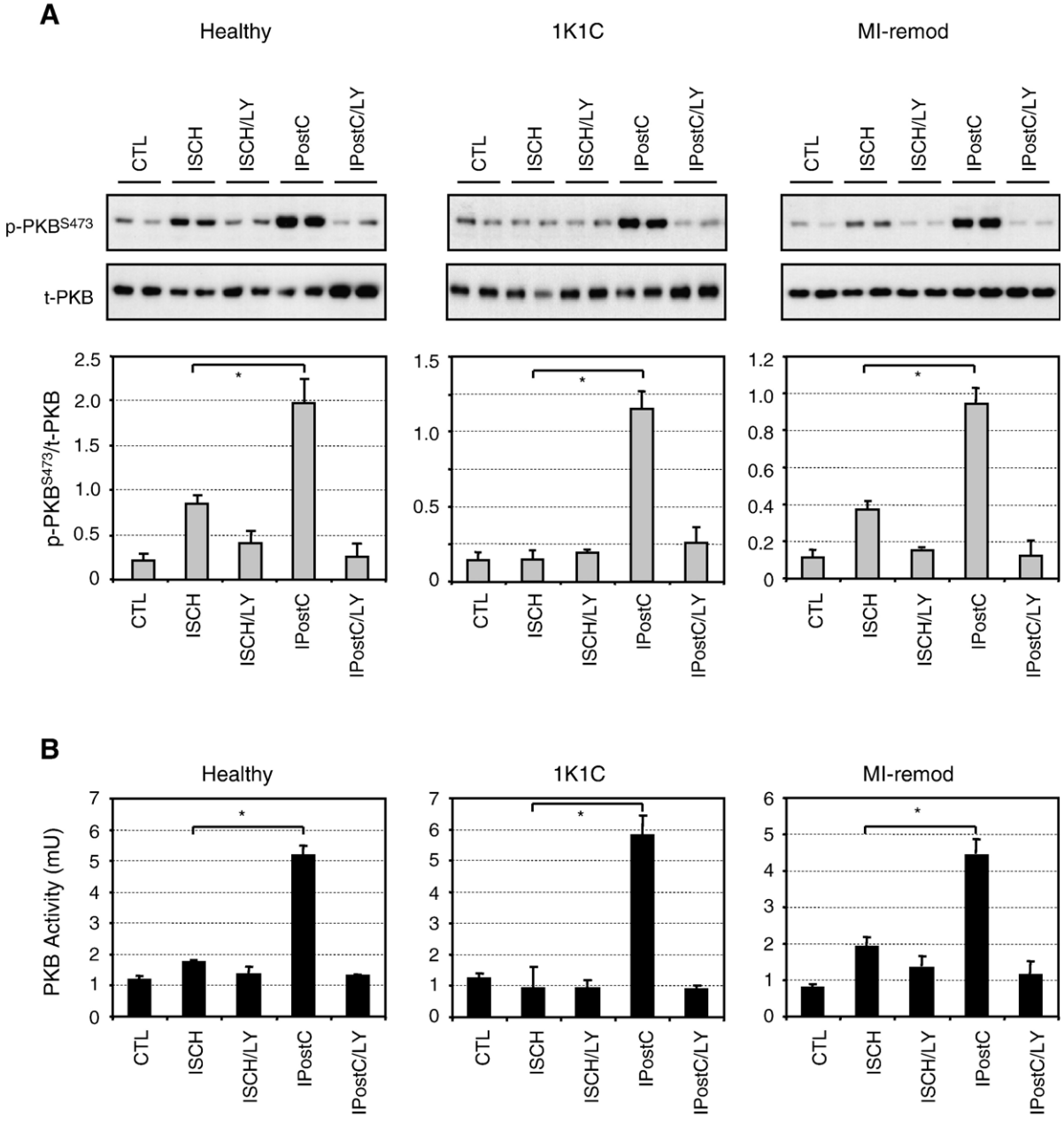

Fig. 3. Phosphorylation status (panel A) and kinase activity (panel B) of protein kinase B/Akt (PKB). Representative Western blots and average density ratio of p-PKB^{S473}/total-PKB for each group. Data are mean±S.D. (n=4 per group). *p<0.05 vs. ISCH.

autoradiography or ^{32}P determination in excised gel slices. One unit of activity was defined as the amount of enzyme that transferred 1 pmol phosphate/min to the substrate at 30 °C.

2.7. Statistics

Data are presented as mean±S.D. For the cardiac functional data, repeated-measures analysis of variance was

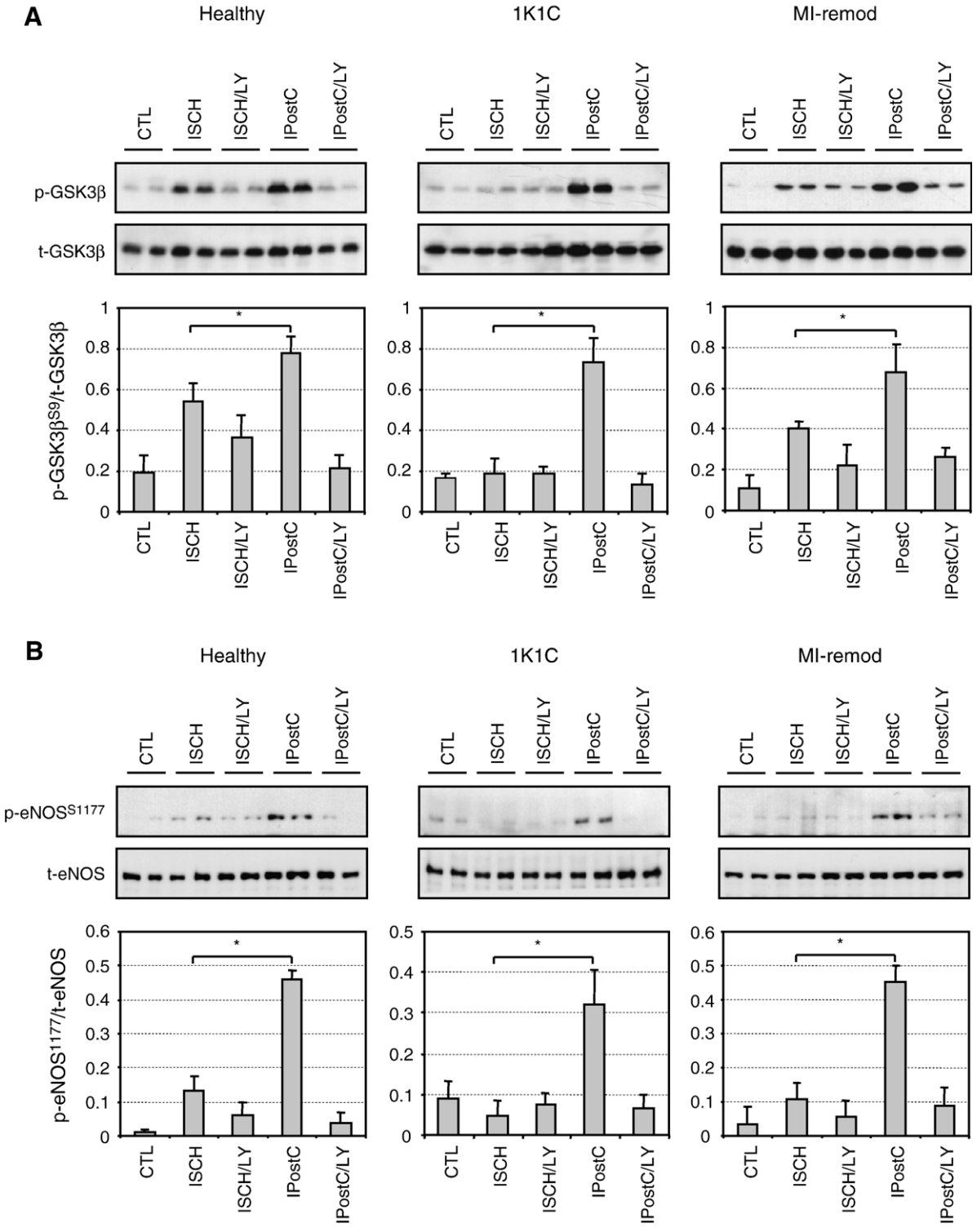

Fig. 4. Phosphorylation status of glycogen synthase kinase3β (GSK3β) (panel A). Representative Western blots and average density ratio of p-GSK3β^{S9}/total-GSK3β. Phosphorylation status of endothelial nitric oxide synthase (eNOS) (panel B). Representative Western blots and average density ratio of p-eNOS^{S1177}/total-eNOS. Data are mean±S.D. (n=4 per group). *p<0.05 vs. ISCH.

used to evaluate differences over time between groups. Unpaired *t*-test was used to compare groups at identical time points, and paired *t*-test to compare within groups over time. *p* values were multiplied by the number of comparisons (Bonferroni correction). Tukey's post-hoc test was applied for multiple comparisons of the one-way analysis of variance for all other data. $p<0.05$ was considered as significant. SigmaStat (version 2.0; SPSS Science, Chicago, IL) was used for the analyses.

3. Results

3.1. Characterization of remodeling and hypertrophy in diseased hearts

Heart- (HW) and body- (BW) weight as well as their ratio (HW/BW) are summarized in Table 1 for the two disease models. HW and HW/BW were significantly higher in the 1K1C and the MI-remodeled (permanent coronary artery ligation) groups, as compared to the healthy and sham groups indicating cardiac hypertrophy in both disease models. Table 1 also shows LVDP, CF, and heart rate (HR) *ex vivo* at equilibration on the Langendorff apparatus. LVDP was significantly higher in the 1K1C and lower in the MI-remodeled groups than in healthy or sham controls, while CF and HR were unchanged. The higher LVDP observed at the Langendorff apparatus corresponds to the significantly ($p<0.001$) higher systolic blood pressure measured *in vivo* in 1K1C (186±18 mm Hg, $n=30$) compared to sham (1K) (123±10 mm Hg, $n=20$) and healthy age-matched animals (120±9 mm Hg, $n=10$). The post-infarct remodeling in MI-remodeled hearts has been characterized in detail in our previous study [8]. The 1K1C hearts displayed an increase in mRNA of about 10-fold for ANP and 4-fold for α-skl-actin, which was accompanied by an increase in protein levels as well (Supplementary Fig. S1A and B). Micrographs of cross-sections of the left ventricular free wall at the mid-ventricular level of 1K1C and MI-remodeled hearts displayed enlarged myocytes in comparison to sham controls (Supplementary Fig. S1C).

3.2. Cardioprotection by ischemic postconditioning is preserved in remodeled myocardium

IPostC significantly improved functional recovery and decreased infarct size in both the 1K1C as well as the MI-remodeled hearts, when compared to unprotected remodeled hearts (Fig. 2A–F and Supplementary Tables 1–3). IPostC achieved over 80% reduction in infarct size and LDH release in MI-remodeled and healthy hearts, while in 1K1C hearts the reduction of both parameters was somewhat lower (69%) (Fig. 2A and B). With regard to hemodynamics, the recovery of LVDP was lower in 1K1C hearts as compared to MI-remodeled and healthy controls after application of IPostC (Fig. 2C). On the other hand, the rate of pressure rise (+d*p*/d*t*, inotropy) was fully preserved in 1K1C, while it was lower by 38% in MI-remodeled hearts (Fig. 2D). The baseline values of LVDP and inotropy were significantly lower in MI-remodeled

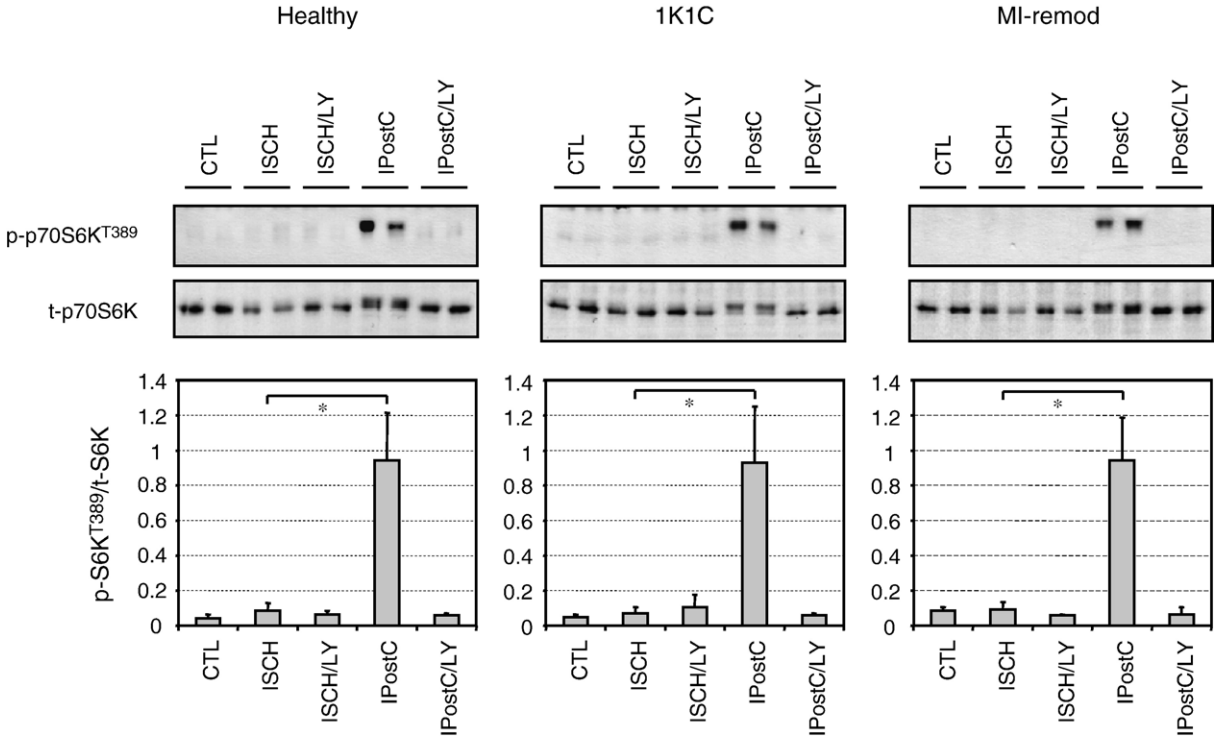

Fig. 5. Phosphorylation status of ribosomal S6 kinase (p70S6K). Representative Western blots and average density ratio of p-p70S6K^{T389}/total-p70S6K. Data are mean±S.D. ($n=4$ per group). *$p<0.05$ vs. ISCH.

hearts, indicating impaired basal cardiac function (Supplementary Table 3). During reperfusion, LVEDP was increased and lusitropy was decreased in 1K1C hearts compared to healthy hearts, indicating impaired postischemic diastolic function in 1K1C hearts. The protection by IPostC was completely abolished by co-administration of the PI3K inhibitor LY294002 (Fig. 2A–F, and Supplementary Tables 1–3). LY294002 or the solvent dimethyl sulfoxide alone administered during reperfusion did not affect functional recovery or infarct size (data not shown). These results provide strong evidence that PI3K-dependent protection by IPostC is preserved and operative in both disease models.

3.3. Ischemic postconditioning in remodeled myocardium activates PKB/Akt and its downstream targets GSK3β, eNOS, and p70S6K in a PI3K-dependent manner

Activation of the pro-survival PI3K–PKB/Akt signaling pathway has been shown to be important for cardioprotection in several healthy animal models [4,5,11–14]. Here we show that IPostC significantly increased phosphorylation of PKB/Akt (Fig. 3A) and its downstream targets GSK3β, eNOS, and p70S6K in remodeled hearts (Figs. 4 and 5). IPostC-induced phosphorylation of PKB/Akt and its downstream substrates GSK3β, eNOS, and p70S6K was suppressed by

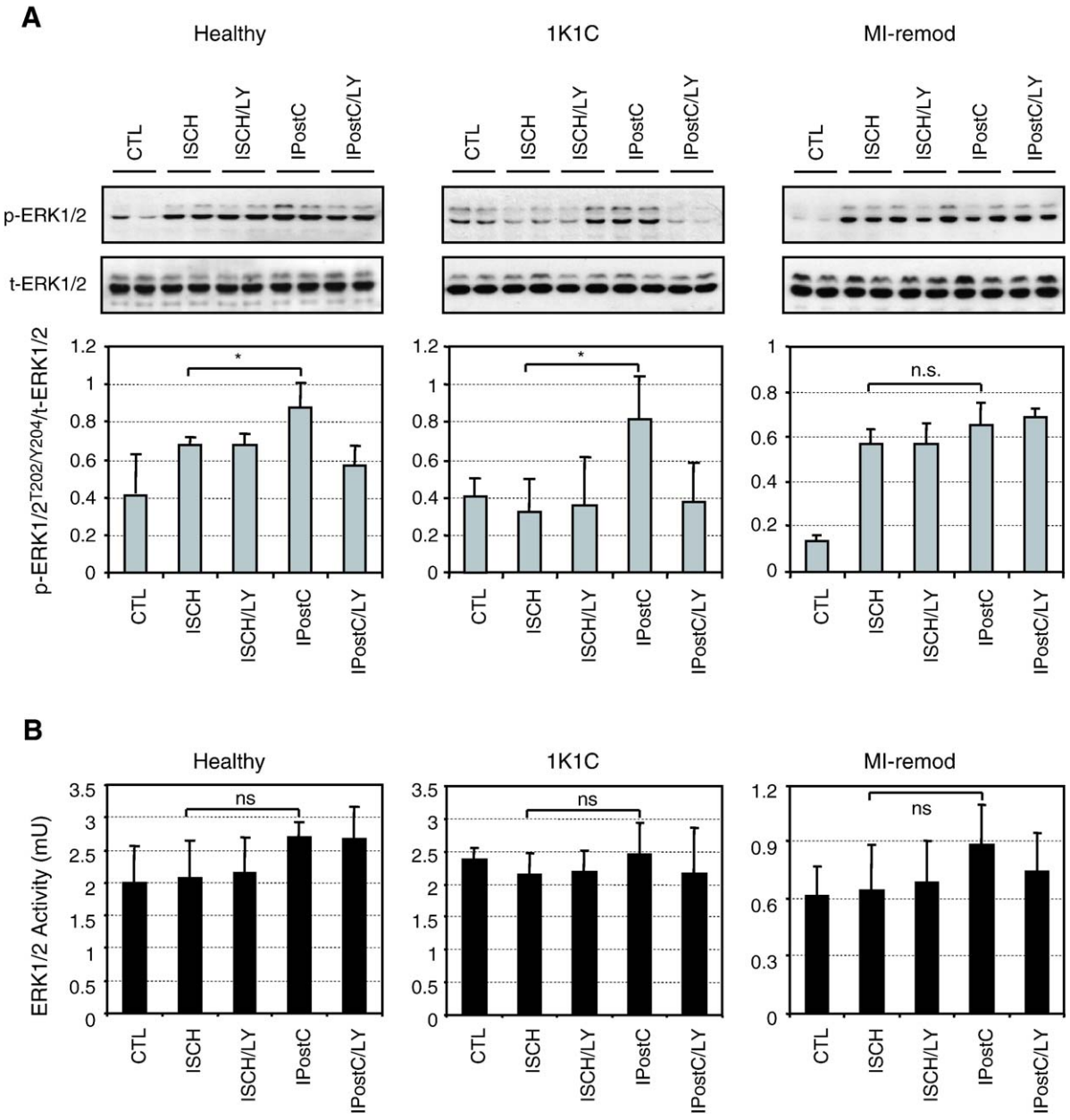

Fig. 6. Phosphorylation status (panel A) and kinase activity (panel B) of extracellular-signal regulated kinase 1/2 (ERK1/2). Representative Western blots and averaged density ratio of p-ERK1/2$^{T202/Y204}$/total-ERK1/2 for each group. Data are mean±S.D. (n=4 per group). *p<0.05 vs. ISCH.

LY294002. Dimethyl sulfoxide alone had no effect on the phosphorylation of PKB/Akt when compared to the ischemia group (data not shown). To further characterize the activation status of PKB/Akt, an *in vitro* kinase assay was carried out. Consistent with the results from Western blots, the activity of PKB/Akt was significantly elevated by IPostC compared to the ischemic control and completely abolished by LY294002 (Fig. 3B). Phosphorylation and enzyme activity of PKB/Akt were partially increased by ischemia alone compared to the time-matched controls in both healthy and MI-remodeled hearts. However, this was not observed in the 1K1C hearts (Fig. 3A and B). Taken together, the data underscore the unique role of PKB/Akt signaling in the IPostC-mediated protection of the remodeled myocardium.

3.4. Cardioprotection by ischemic postconditioning in the remodeled myocardium does not primarily depend on ERK1/2 signaling

Beside PI3K–PKB/Akt, ERK1/2 signaling presents another component in the RISK pathways that has also been suggested to contribute to the reduction of infarct size with postconditioning [13]. We therefore examined whether the protection by IPostC would be associated with activation of the ERK1/2 pathway in the remodeled hearts. In healthy hearts, phosphorylation of ERK1/2 was moderately increased ($p=0.033$) by ischemia alone, and was further enhanced by IPostC (Fig. 6A). In 1K1C hearts, phosphorylation of ERK1/2 was not increased by ischemia alone but was increased by IPostC (Fig. 6A). The elevated phosphorylation of ERK1/2 by IPostC was reversed by LY294002 suggesting a PI3K-dependent phosphorylation of ERK1/2. In contrast, phosphorylation of ERK1/2 in the MI-remodeled hearts was strongly increased by ischemia alone, but was resistant to LY294002 and not further increased by IPostC, indicating that a PI3K-independent mechanism was responsible for the ischemia-induced ERK1/2 phosphorylation in the MI-remodeled hearts. These results based on the phosphorylation status of ERK1/2 suggest that the ERK1/2 pathway is differentially regulated in the two types of remodeling. In parallel, the activities of ERK1/2 were also determined by an *in vitro* kinase assay. Surprisingly, there was no increase in ERK1/2 activities by IPostC in both remodeling models as well as in healthy hearts (Fig. 6B). Moreover, the activities of ERK1/2 in the MI-remodeled hearts were 3-fold lower than that in healthy and 1K1C hypertrophied hearts, suggesting that subtle differences in the regulation of RISK pathways may exist between the models. Collectively, our results based on direct enzyme activity measurements indicate that the ERK1/2 pathway is not primarily involved in IPostC-mediated protection in both healthy and diseased hearts.

4. Discussion

Here we show for the first time that protection by IPostC is preserved in two rat models of myocardial remodeling. IPostC was previously reported to be ineffective in limiting infarct size in rabbits with hypercholesterolemia and atherosclerosis [15]. However, our results are in accordance with a most recent clinical trial by Staat and colleagues [16], who postconditioned hearts of patients undergoing percutaneous coronary interventions with four episodes of 1-min balloon inflations starting within 1 min of reflow. Using creatine kinase release as a surrogate marker of infarct size, these authors found that myocardial damage could be reduced by 36%.

Myocardial remodeling is a short-term adaptive but long-term maladaptive process to a variety of hemodynamic conditions associated with increased cardiac work. Characteristic structural changes [17], alterations in metabolism [18], and cellular signaling [19–21] put the remodeled myocardium at particular risk for further ischemic damage. Since most individuals who experience acute ischemic heart disease have underlying myocardial remodeling, we addressed the important question whether the diseased remodeled myocardium is still receptive to protection by IPostC using two experimental models of remodeling. While 1K1C remodeled hearts develop eccentric hypertrophy by volume overload [10], MI-remodeled hearts display a far more complex architectural rearrangement due to (i) loss of viable tissue and scar formation (ii) volume overload by scar expansion (iii) pressure overload induced by increasing volume overload [20]. Nonetheless, hypertrophy is a hallmark of both types of remodeling. Since these structural changes are associated with alterations in neurohormonal activation and cellular signaling, we hypothesized that remodeling may abolish innate protective mechanisms and render the heart more susceptible to ischemia.

Previous studies identified the survival kinases PI3K–PKB/Akt and ERK1/2 as key players in the protection afforded by IPostC in healthy myocardium [6]. However, the relative importance of the two kinases in mediating the protection remains controversial. A study investigating pharmacological postconditioning by 5′-(*N*-ethylcarboxamido) adenosine and bradykinin suggests that PKB/Akt is upstream of ERK1/2 [5], while results from a more recent study stress the pivotal role of ERK1/2 but not PI3K–PKB/Akt in IPostC-mediated protection [13]. Moreover, Schwartz et al. [14] reported that IPostC activates both PKB/Akt and ERK1/2 but yet failed to protect against ischemic injury in pigs. In the present study, we show that PKB/Akt is activated and that its downstream targets GSK3β, eNOS, and p70S6K are markedly phosphorylated by IPostC not only in the healthy but also in the remodeled hearts. This phosphorylation is commensurate with functional and structural protection and is sensitive to inhibition by LY294002. Importantly, our results on PKB/Akt activation are not solely based on Western blot analysis using phosphor-specific antibodies, but further rely on *in vitro* kinase assays, which directly measure catalytic activity. Although we observed an increase in ERK1/2 phosphorylation by IPostC in healthy and 1K1C hearts, the directly measured catalytic activity of ERK1/2 was not elevated by IPostC in healthy and diseased hearts.

Obviously, the observed phosphorylation of ERK1/2 by IPostC was not sufficient to protect. Hence our data suggest that PI3K–PKB/Akt but not ERK1/2 is the predominant mediator of IPostC-induced protection in both healthy and diseased hearts. Of note, our study is the first to measure ERK1/2 activation in IPostC by assessing the phosphorylation status in conjunction with *in vitro* activity measurements. Earlier studies on ERK1/2 activity in pre- and postconditioning exclusively relied on Western blotting, which may, at least in part, explain the controversial role of ERK1/2 in IPostC. Nonetheless, our results cannot rule out the possibility of some cross talk between ERK1/2 and PKB/Akt, and the two pathways may also follow different spatio-temporal routes at the onset of reperfusion in the heart. Therefore, additional time-course and blocker experiments will be required to ultimately delineate the role of PKB/Akt and ERK1/2 in IPostC-mediated protection in remodeled myocardium.

We found noteworthy alterations in PKB and ERK1/2 signaling in the different disease models. First, although the maximal activation of PKB/Akt can be achieved by IPostC in all three models, partial activation of PKB/Akt by ischemia alone was lost in 1K1C hearts. This observation may explain the observed reduced protection by IPostC in 1K1C rats. Second, the profiles of ERK1/2 phosphorylation in both types of remodeling and the basal kinase activity of ERK1/2 in MI-remodeled hearts were different from those observed in healthy hearts. These alterations suggest that key players of the RISK pathway may be differentially regulated by different types of cardiac remodeling. Finally, we found that the recovery of inotropy achieved by IPostC was impaired in MI-remodeled hearts, while both recovery of LVDP and infarct size reduction were unaffected. The reason for these differences is unclear. A recent study using a transgenic mouse model of hypertrophy with cardiac-specific expression of myristoylated PKB/Akt demonstrated that activation of PKB/Akt alone is not sufficient for protection [22]. This implies that additional PI3K-dependent but PKB/Akt-independent signaling components may be required for full cardioprotection. Thus, it is possible that such alternative mechanisms are impaired in MI-remodeled hearts.

Ischemic preconditioning has been shown to be ineffective in MI-remodeled rabbit hearts [23], but proved its efficacy in several hypertrophy models [24–27]. Despite these partly promising results, the concept of preconditioning could not be successfully established in the clinical setting. Therefore, recent interventions aimed at modifying reperfusion, but IPostC may further jeopardize the diseased heart. This could be avoided by utilizing pharmacological agents mimicking the biological process of IPostC. Hence, the understanding of the molecular mechanisms underlying IPostC is of paramount importance and should be exploited in additional studies. Importantly, future studies should particularly investigate the effects of the disease-related alterations in signaling on cardioprotection.

In summary, using a highly controlled experimental setting, we show in our study that remodeled rat hearts with hypertrophy induced by infarction (permanent coronary artery ligation) and 1K1C hypertension are still receptive to protection by IPostC. Furthermore, we identified in these models the PI3K–PKB/Akt signaling pathway as predominant mediator of IPostC-induced cardioprotection.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2006.06.027.

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Cardiac remodelling hinders activation of cyclooxygenase-2, diminishing protection by delayed pharmacological preconditioning: role of HIF1α and CREB

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Aims We tested whether delayed pharmacologic preconditioning elicited by isoflurane is protective in infarct-remodelled hearts.

Methods and results Male Wistar rats were treated with the preconditioning drug isoflurane 6 weeks after permanent ligation of the left anterior descending coronary artery. Twenty-four and 48 h later, hearts were perfused on the Langendorff system and treated with cyclooxygenase-2 or 12-lipoxygenase inhibitors before exposure to 40 min of ischaemia followed by 90 min of reperfusion. Infarct size was determined by triphenyltetrazolium chloride staining and lactate dehydrogenase release. Cyclooxygenase-2 expression and activity were measured by Western blotting and colorimetric assay. Nuclear translocation of cyclooxygenase-2-inducing transcription factors HIF1α, CREB, STAT3, and NFκB was determined. Post-infarct, remodelled hearts exhibit alterations in cellular signalling, time course and extent of isoflurane-induced late protection. While remodelled, preconditioned hearts exhibited protection exclusively at 24 h, healthy hearts showed sustained protection for up to 48 h, which correlated with cyclooxygenase-2 protein expression and enzymatic activity. The cyclooxygenase-2 inhibitors celecoxib and NS-398, but not the 12-lipoxygenase inhibitor cinnamyl-3,4-dihydroxycyanocinnamate, abolished delayed protection in both healthy and remodelled hearts, identifying cyclooxygenase-2 as a key mediator of late protection in both models. Isoflurane induced nuclear translocation of HIF1α in all hearts, but CREB was exclusively activated in healthy but not remodelled myocardium, which expressed higher levels of the CREB antagonist ICER. Delayed protection by isoflurane in remodelled hearts was more vulnerable to inhibition by celecoxib.

Conclusion Isoflurane failed to mobilize cyclooxygenase-2-inducing CREB in ICER-overexpressing, remodelled hearts, which was associated with a shortening of the second window of protection.

1. Introduction

In 1993, Marber *et al.*[1] and Kuzuya *et al.*[2] described a remarkable phenomenon called delayed ischaemic preconditioning of the heart, which reflects a late 'window of protection' against ischaemia, usually occurring 12–72 h after brief repetitive ischaemic episodes. The resulting protective phenotype of the heart is due to a profound transcriptional reprogramming with *de novo* protein synthesis, and is of great clinical relevance, as it is 30-times longer than the early window, which only lasts 2–3 h.[3] Today, we know that early and delayed preconditioning of the heart can be elicited more safely by pharmacologic means such as halogenated ethers or opioids than by ischaemic episodes.[4,5] This is of particular relevance for the already jeopardized diseased myocardium. Isoflurane-induced delayed protection was first reported in 2003 in a rabbit model by Toncovic-Capin *et al.*[6] In healthy rabbit myocardium, celecoxib, a specific

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cyclooxygenase-2 (COX-2) inhibitor, abolished delayed isoflurane preconditioning,[7] while late isoflurane protection was dependent on 12-lipoxygenase activity in healthy murine hearts.[8] Recently, molecular evidence of delayed anaesthetic preconditioning after sevoflurane inhalation has been also reported in humans.[9]

Most experimental studies evaluated the phenomenon of early and delayed preconditioning in healthy juvenile hearts, but this is far from clinical reality, as diseased myocardium would benefit most from this protection. Some studies reported reduced protection in the early phase of preconditioning in diseased hearts.[10,11] Since the early phase of preconditioning is based on similar signalling pathways as the late phase, it could be speculated that protection during the late phase would be impaired in remodelled hearts. Post-infarct remodelled myocardium exhibits marked structural changes,[12] alterations in energy metabolism[13] and cellular signalling,[14] which put the heart at particular risk for further ischaemic damage. In contrast to this notion are our own recent findings that post-infarct remodelled hearts remain receptive to protection by early isoflurane preconditioning via the same signalling pathways, as observed in healthy hearts.[15] Nonetheless, no data exist as to whether delayed anaesthetic protection retains its effectiveness in post-infarct myocardium. Using an established rat model of ventricular remodelling after permanent coronary artery ligation, we investigated whether ventricular remodelling would affect late isoflurane protection. Specifically, we hypothesized that remodelling would abolish late isoflurane protection and increase the vulnerability of the heart to ischaemia-reperfusion injury. This study also investigated possible mechanisms underlying the postulated refractoriness to late anaesthetic protection in remodelled hearts.

The current study shows that the isoflurane-induced second window of protection shrinks to a short-lived phase in remodelled myocardium, which would require repetition of the preconditioning stimulus every 24 h to maintain the heart in the protected state.

2. Methods

This animal study was performed according to the guidelines of the Animal Care and Use Committee of the University of Zurich, Switzerland. All experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 85-23, revised 1996).

2.1 Post-infarct remodelled hearts

Myocardial infarction ($\sim$35% of left ventricular mass) and subsequent hypertrophic remodelling was induced in male adult (180–200 g, 8–9 weeks old) Wistar rats by permanent ligation of the left anterior descending coronary artery (LAD) under anaesthesia, as previously described in detail.[16] Some animals served as control and underwent the same procedure except that the suture was passed under the coronary artery without ligation. Rats were sacrificed 6 weeks after surgery, and the body weight and heart weight were measured. Hearts were further evaluated for their function on a Langendorff apparatus (see Supplementary material online, *Table S1*).

2.2 Protocol for delayed isoflurane preconditioning

Healthy age-matched rats and infarct rats 6 weeks after coronary artery ligation were placed on a thermoregulating pad and exposed to 2.1 vol% [1.5 minimum alveolar concentration (MAC) in rats] isoflurane (Abbott, Bar, Switzerland) in oxygen for 90 min by inhalation (*Figure 1*). Rats exposed to 100% oxygen for 90 min served as sham control. The concentration of applied isoflurane was continuously monitored using a gas monitor. In separate experiments, a catheter was inserted in the femoral artery and arterial blood was withdrawn for blood gas analysis (see Supplementary material online, *Table S2*). The animals subsequently recovered in room air (21 vol% oxygen). Twenty-four and 48 h later, delayed protection against ischaemia-reperfusion injury was assessed on a Langendorff apparatus (*Figure 1*).

2.3 Isolated perfused rat heart experiments

Rats were heparinized (500 units i.p.) and 15 min later decapitated without prior anaesthesia. Hearts were mounted on a non-circulating Langendorff apparatus and perfused with Krebs–Henseleit buffer gassed with 95% O_2 and 5% CO_2 at pH 7.4 and 37°C, as previously described.[17] Five hearts were assigned to each experimental group (*Figure 1*). The selective COX-2 inhibitors celecoxib (CEL, Pfizer AG, Zurich, Switzerland, $IC_{50} = 0.04$ μM, low dose used 0.1 μM, high dose used 1 μM), N-2-cyclohexyloxy-4-nitrophenyl-methanesulphonamide (NS-398, Cayman Chemical, Ann Arbor, MI, $IC_{50} = 1.77$ μM, used concentration 5 μM) and the selective 12-lipoxygenase inhibitor cinnamyl-3,4-dihydroxycyanocinnamate (CDC, USBiological, Swampscott, MA, $IC_{50} = 0.063$ μM, used concentration 0.5 μM) were dissolved in 0.1% dimethyl sulphoxide (DMSO) vehicle and used to perfuse the hearts for 10 min before induction of global ischaemia (*Figure 1*). Infarct size was determined by 1% 2,3,5-triphenyltetrazolium chloride staining after 90 min of reperfusion, as previously described.[16,18] In addition, myocardial damage was estimated by measuring the release of lactate dehydrogenase (LDH) from necrotic tissue, as previously described.[16] Briefly, the perfusate was collected and LDH activity was determined by the Roche/Hitachi 917 kit (sensitivity 6 U/L, intra- and interassay coefficients of variance $<$1%).

2.4 Western blot analysis

Separate experiments served to determine expression of 12-lipoxygenase, 5-lipoxygenase, and COX-2 in healthy and remodelled hearts 24 and 48 h after isoflurane exposure by Western blot analysis. The antibodies were from the following sources: COX-2, Cell Signaling Technology, Beverly, CA, USA; 12-lipoxygenase, Cayman Chemical; 5-lipoxygenase, BD Biosciences, San Diego, CA, USA; α-tubulin, Sigma, St Louis, MO, USA.

2.5 COX-2 activity assay

Left ventricular tissue was homogenized in cold homogenization buffer (0.1 M Tris-HCl, pH 7.8 containing 1 mM EDTA). After centrifugation at 10 000 g for 20 min, the supernatant was collected for enzyme assay. COX-2 activity was measured in the presence of a potent and selective cyclooxygenase-1 inhibitor SC-560 (Cayman Chemical, IC_{50} for cyclooxygenase-1 = 9 nM and for cyclooxygenase-2 =

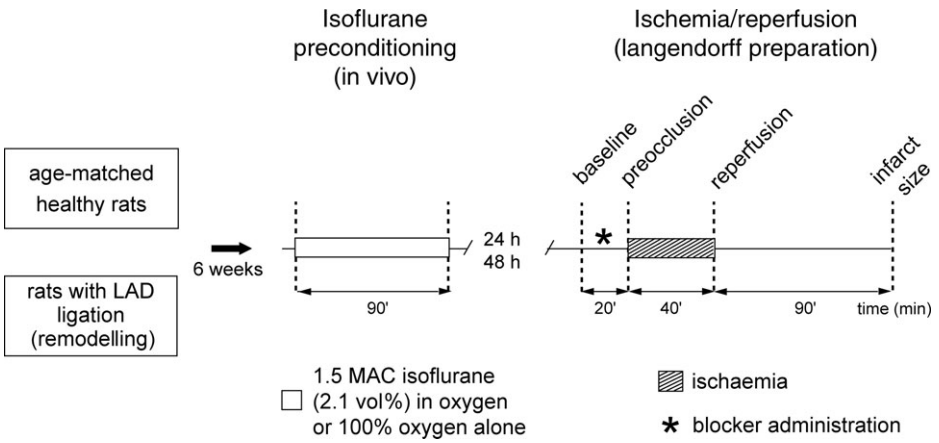

Figure 1 Treatment protocols. Six weeks after ligation of the left anterior descending coronary artery (LAD), rats were exposed *in vivo* to 2.1 vol% (1.5 MAC) isoflurane in oxygen or oxygen alone for 90 min. Age-matched healthy rats served as control group. Twenty-four and 48 h after isoflurane preconditioning, Langendorff perfused healthy and remodelled hearts were exposed to 40 min of ischaemia and 90 min of reperfusion. Blockers were administered 10 min before index ischaemia. MAC: minimum alveolar concentration.

6.3 μM) using a chromogenic cyclooxygenase activity assay kit according to the manufacturer's instruction (Cayman Chemical). This assay measures the colour reaction of cyclooxygenase-generated hydroperoxides.[19]

2.6 Electrophoretic mobility shift assays

Nuclear proteins were extracted as previously described in detail.[20] Electrophoretic mobility shift assays (EMSAs) were performed with Odyssey® IRDye® 700 infrared dye labelled double-stranded oligonucleotides coupled with the EMSA buffer kit (LI-COR Bioscience, Bad Homburg, Germany) according to the manufacturer's instructions. Briefly, 10–20 μg of nuclear extract was incubated with 1–1.5 μL of IRDye® 700 infrared dye labelled oligonucleotides, 2 μL of 10× binding buffer, 2.5 mM DTT, 0.25% Tween, and 1 μg of poly (dI-dC) in a total volume of 20 μL for 30 min at room temperature. For CREB binding assay a final concentration of 0.05% NP-40 was added, and for STAT3 binding assay a final concentration of 0.05% NP-40 and 5 mM MgCl were added. Samples were separated on a 4% polyacrylamide gel in 0.5× tris-borate-EDTA running buffer for 2 h at 200 V. The gel was then scanned by direct infrared fluorescence detection on the Odyssey® Imaging System (LI-COR Bioscience). Oligonucleotide sequences of the double-stranded DNA probes used were as follows: HIF-1α, 5′-AGC TTG CCC TAC GTG CTG TCT CAG A-3′; CREB, 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′; NFκB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; STAT3, 5′-GAT CCT TCT GGG AAT TCC TAG ATC-3′. The specificity of the DNA-protein binding signal was confirmed by competition with 200-fold molar excess of the unlabelled consensus competitor oligonucleotide.

2.7 Real-time quantitative polymerase chain reaction

Details are provided in the Supplementary Materials and Methods.

2.8 Statistical analysis

Data are presented as mean ± SD. For cardiac functional data, repeated-measures analysis of variance was used to evaluate differences over time between groups. Unpaired *t*-test was used to compare groups at identical time points, and paired *t*-test to compare within groups over time. *P*-values were multiplied by the number of comparisons (Bonferroni correction). Post-hoc Tukey test was applied for multiple comparisons of the one-way analysis of variance for all other data. $P < 0.05$ was considered as significant. SigmaStat (version 2.0; SPSS Science, Chicago, IL, USA) was used for the analyses.

3. Results

3.1 Post-infarct remodelling narrows the second window of protection after isoflurane preconditioning

From the 106 rats used for coronary artery ligation, 10 animals were lost intraoperatively due to intractable ventricular fibrillation. Heart weight over body weight ratios determined 6 weeks after surgery were markedly higher in post-infarct hearts compared with age-matched healthy or sham-operated hearts (remodelled: 5.52 ± 0.70 g/kg, healthy: 3.58 ± 0.57 g/kg, sham-operated: 3.63 ± 0.55 g/kg, $P < 0.05$; see Supplementary material online, *Table S1*), indicating significant ventricular remodelling. In addition, left ventricular developed pressure, coronary flow, and heart rate were determined *ex vivo* on the Langendorff apparatus. Left ventricular developed pressure was lower in remodelled hearts compared with age-matched healthy hearts. No changes were observed between groups for coronary flow and heart rate (see Supplementary material online, *Table S1*).

Preconditioning elicited by isoflurane inhalation at 1.5 MAC for 90 min significantly improved functional recovery (see Supplementary material online, *Table S3 and S4*) and decreased infarct size after ischaemia-reperfusion in both healthy and remodelled hearts (*Figures 2A* and *3A*). However, protection in infarct hearts was exclusively present at 24 h after preconditioning, while in healthy hearts the protection extended from 24 to 48 h after isoflurane exposure. Infarct-size reduction was corroborated with measurements of LDH release into the perfusate during reperfusion in healthy and remodelled hearts (*Figures 2B*

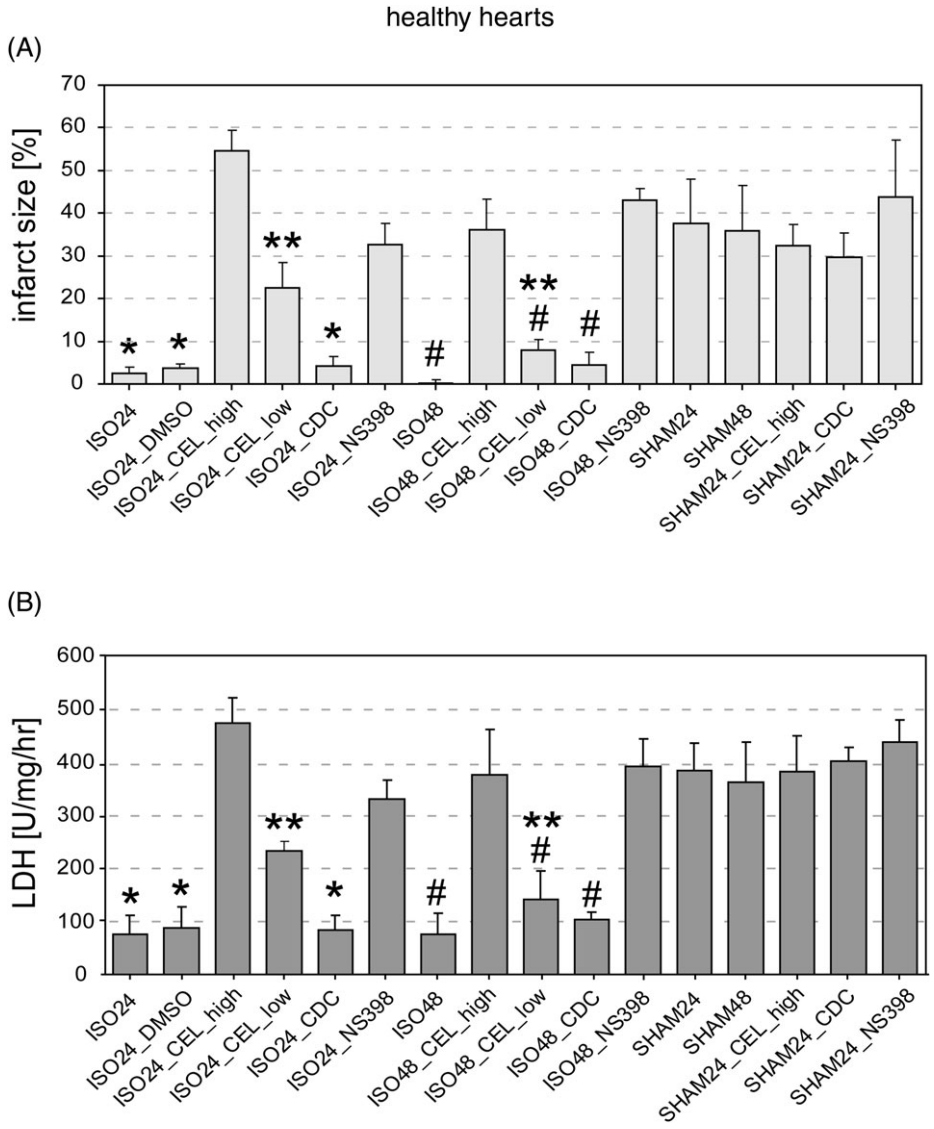

Figure 2 Infarct size (*A*) and lactate dehydrogenase (LDH) release (*B*) in healthy hearts. ISO24/48: hearts exposed to ischaemia-reperfusion 24 or 48 h after isoflurane preconditioning. ISO24/48_CEL_high: preconditioned hearts treated with 1 μM celecoxib. ISO24/48_CEL_low: preconditioned hearts treated with 0.1 μM celecoxib. ISO24/48_CDC: preconditioned hearts treated with 0.5 μM cinnamyl-3,4-dihydroxy-cyanocinnamate (CDC). ISO24/48_NS398: preconditioned hearts treated with 5 μM N-2-cyclohexyloxy-4-nitrophenyl-methane-sulphonamide (NS-398). Final concentration of dimethyl sulphoxide (DMSO) was <0.1%. The prefix SHAM in group names indicates respective groups without isoflurane preconditioning (oxygen alone). $^{*}P < 0.05$ vs. SHAM24 groups. $^{\#}P < 0.05$ vs. SHAM48 groups. $^{**}P < 0.05$ vs. high celecoxib concentration. Data are mean ± SD ($n = 5$ per group).

and *3B*). Sham groups (oxygen inhalation alone) did not exhibit protection 24 or 48 h later in either model. The recovery of developed pressure after delayed preconditioning was lower by ~20% in remodelled hearts when compared with healthy hearts, and end-diastolic pressure reached baseline values after 90 min of reperfusion in healthy but not remodelled hearts (see Supplementary material online, *Tables S3 and S4*). Groups with celecoxib administration before ischaemia-reperfusion exhibited increased coronary flow consistent with previous reports.[21] Of note, during triggering of isoflurane preconditioning, arterial blood gas measurements did not show significant differences compared with animals treated with oxygen alone (see Supplementary material online, *Table S2*). These results provide evidence that post-infarct remodelling narrows the second window of protection after isoflurane preconditioning.

3.2 Late protection by isoflurane preconditioning is more vulnerable to cyclooxygenase-2 inhibition in remodelled myocardium

COX-2 and 12-lipoxygenase were shown to be important in mediating delayed cardioprotection by isoflurane.[7,8] Here we show that delayed protection by isoflurane is abolished or attenuated by inhibitors of COX-2, but not of 12-lipoxygenase (*Figures 2* and *3*). While delayed protection was more resistant to celecoxib in healthy hearts, remodelled hearts completely lost protection in the presence of even lower celecoxib concentrations (see Supplementary material online, *Tables S3 and S4*). Dimethyl sulphoxide alone or blockers alone had no effect on infarct size. Taken together, the data provide evidence that delayed protection by isoflurane is mediated by COX-2 in both healthy and remodelled hearts and further underscore the

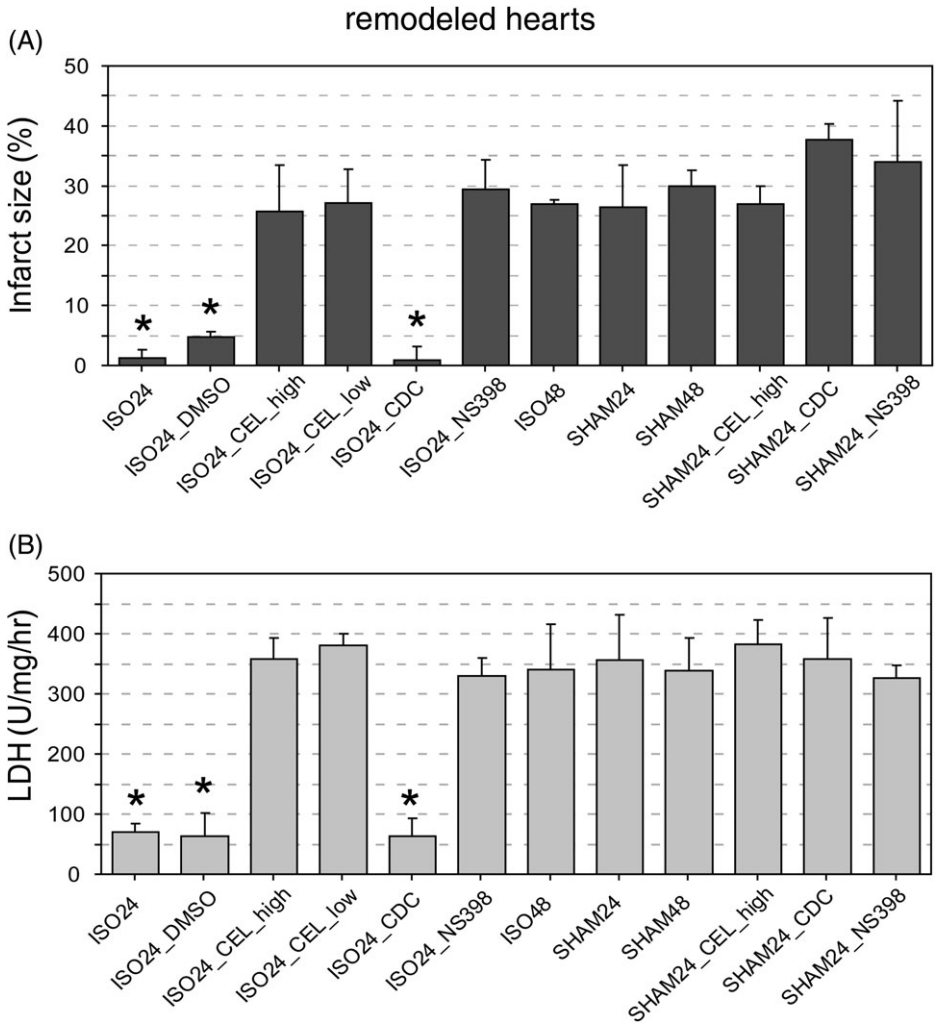

Figure 3 Infarct size (*A*) and lactate dehydrogenase (LDH) release (*B*) in remodelled hearts. ISO24/48: hearts exposed to ischaemia-reperfusion 24 or 48 h after isoflurane preconditioning. ISO24/48_CEL_high: preconditioned hearts treated with 1 μM celecoxib. ISO24/48_CEL_low: preconditioned hearts treated with 0.1 μM celecoxib. ISO24/48_CDC: preconditioned hearts treated with 0.5 μM cinnamyl-3,4-dihydroxy-cyanocinnamate (CDC). ISO24/48_NS398: preconditioned hearts treated with 5 μM N-2-cyclohexyloxy-4-nitrophenyl-methane-sulphonamide (NS-398). Final concentration of dimethyl sulphoxide (DMSO) was $<0.1\%$. The prefix SHAM in group names indicates corresponding groups without isoflurane preconditioning (oxygen alone). $^{*}P < 0.05$ vs. SHAM24 group. Data are mean $\pm$ SD ($n = 5$ per group).

remarkable vulnerability of the diseased myocardium to COX-2 inhibition.

3.3 Isoflurane-induced cyclooxygenase-2 expression and activity show alterations in remodelled hearts

As expected, COX-2 was more abundant in infarct hearts (see Supplementary material online, *Figure S1*).[22] To characterize the expression and activity of COX-2 in delayed protection by isoflurane preconditioning, Western blot analyses (*Figure 4*) and activity measurements (*Figure 5*) were performed in isoflurane- and sham-treated rat hearts 24 and 48 h after triggering with isoflurane. In healthy hearts, COX-2 expression was increased 24 (1.5-fold) and 48 h (2.7-fold) after isoflurane exposure, while in remodelled hearts, COX-2 expression was exclusively increased at 24 h (three-fold) (*Figure 4*). Oxygen inhalation in sham groups did not affect COX-2 expression or activity. COX-2 activity closely paralleled COX-2 expression (*Figure 5*). No changes in 12-lipoxygenase and 5-lipoxygenase expression were observed (see Supplementary material online, *Figure S2*). Together, isoflurane-induced COX-2 showed a shortened activation profile in remodelled hearts, which closely paralleled structural and functional protection.

3.4 Isoflurane induces nuclear translocation of HIF1α but not CREB in post-infarct remodelled hearts

To determine the contribution of putative transcription factors in the observed isoflurane-induced COX-2 expression in healthy and remodelled hearts, nuclear translocation of NFκB, STAT3, HIF1α, and CREB was measured 30 min after termination of isoflurane inhalation (2.1 vol% for 90 min) in additional experiments. Isoflurane induced nuclear translocation of HIF1α in healthy and remodelled hearts (*Figure 6*). In contrast, CREB was exclusively translocated to nuclei in healthy hearts. None of the other transcription factors was activated by isoflurane compared with sham

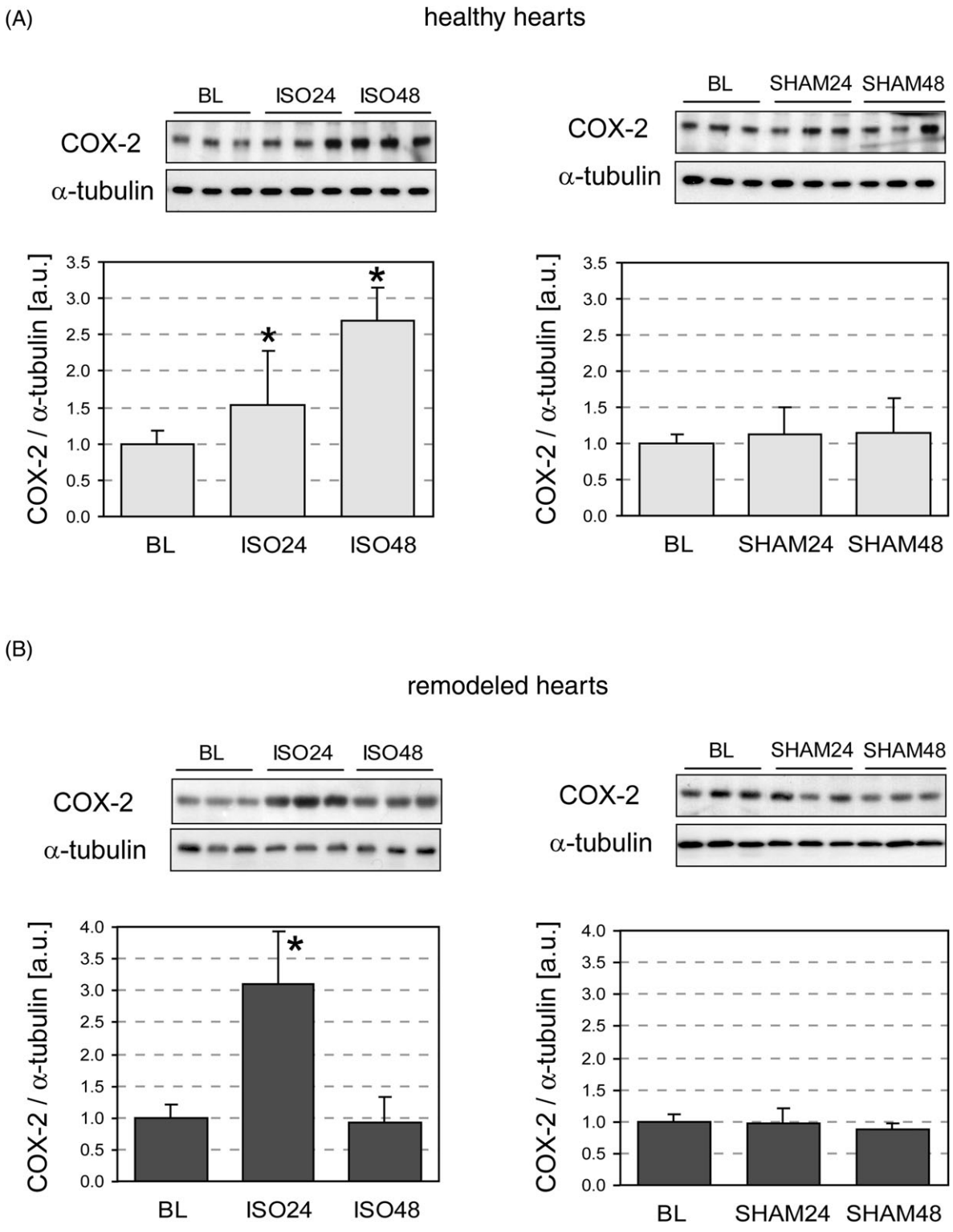

Figure 4 Representative Western blots of cyclooxygenase-2 (COX-2) expression (72 kDa) in healthy (*A*) and remodelled (*B*) hearts 24 and 48 h after isoflurane exposure expressed as average density ratio of COX-2 to α-tubulin (57 kDa) normalized to baseline (BL). ISO24/48: hearts exposed to isoflurane and harvested 24 or 48 h later. SHAM24/48: time-matched hearts exposed to oxygen alone. Data are mean ± SD ($n = 4$ per group). *$P < 0.05$ vs. BL.

treatment (data not shown). In search of possible mechanisms for the lack of CREB activation by isoflurane in remodelled myocardium, we determined the mRNA level of the CREB-antagonist inducible cAMP early repressor (ICER), which was increased by 63% in remodelled myocardium: 0.026 ± ;0.005 vs. 0.016 ± ;0.004 (arbitrary units relative to α-tubulin, $P = 0.009$). These data suggest that the failure of isoflurane to activate CREB in remodelled myocardium may be due to overexpression of the CREB-antagonist ICER in infarct hearts and thus may contribute to the observed narrowing of the second window of protection.

4. Discussion

The salient findings of this study can be summarized as follows. First, isoflurane induced a robust but narrow second window of protection in hearts with marked post-infarct ventricular remodelling. In these diseased hearts, reduction in infarct size and improved functional recovery were only present 24 h after isoflurane application, while in healthy hearts protection extended for up to 48 h. Secondly, delayed protection by isoflurane in rat hearts was mediated by COX-2 but not 12-lipoxygenase in both remodelled and healthy myocardium, and closely correlated with increased COX-2 expression and activity levels. Hence, isoflurane elicits late protection in remodelled rat myocardium via similar signalling pathways as previously reported in healthy rabbit hearts.[7] While COX-2-inducing HIF-1α was activated by isoflurane in healthy and remodelled hearts, isoflurane failed to mobilize COX-2-inducing CREB in post-infarct myocardium. Overexpression of the CREB-antagonist ICER offers an explanation for the observed narrowing of the second window of protection, a hypothesis that needs validation. Thirdly, despite increased expression of COX-2, remodelled preconditioned hearts exhibited a remarkable vulnerability to inhibition by the clinically used anti-inflammatory drug celecoxib, which completely abolished delayed isoflurane protection in these hearts, but only partly diminished the protection in healthy hearts. Together, we present for the first time evidence that delayed protection by isoflurane preconditioning varies with the disease state of the heart and concomitant medication (*Figure 7*). Since recruitment of COX-2 is a critical innate mechanism, whereby the heart protects itself from ischaemia,[3] our findings may be of relevance for the medical management of patients at risk of cardiovascular complications.

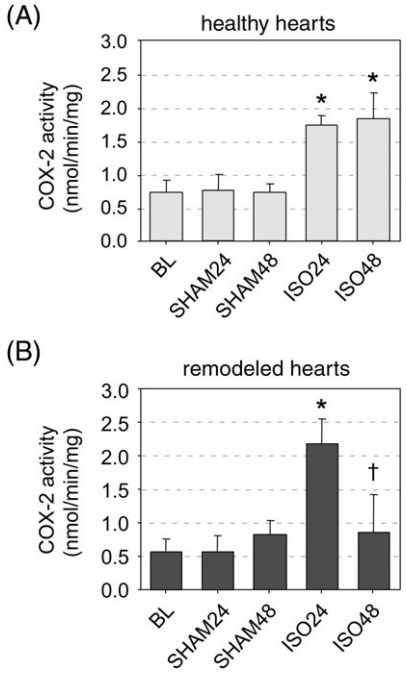

Figure 5 Cyclooxygenase-2 (COX-2) activity in healthy (*A*) and remodelled (*B*) hearts 24 and 48 h after isoflurane exposure. ISO24/48: hearts exposed to isoflurane and harvested 24 or 48 h later. SHAM24/48: time-matched hearts exposed to oxygen alone. Data are mean $\pm$ SD ($n = 4$ per group). *$P < 0.05$ vs. baseline (BL).

Delayed preconditioning is a second window of protection whereby stimulation by ischaemia or chemical agents enhances the resistance of the heart to subsequent potentially lethal stimuli 24–72 h later. It is a universal response

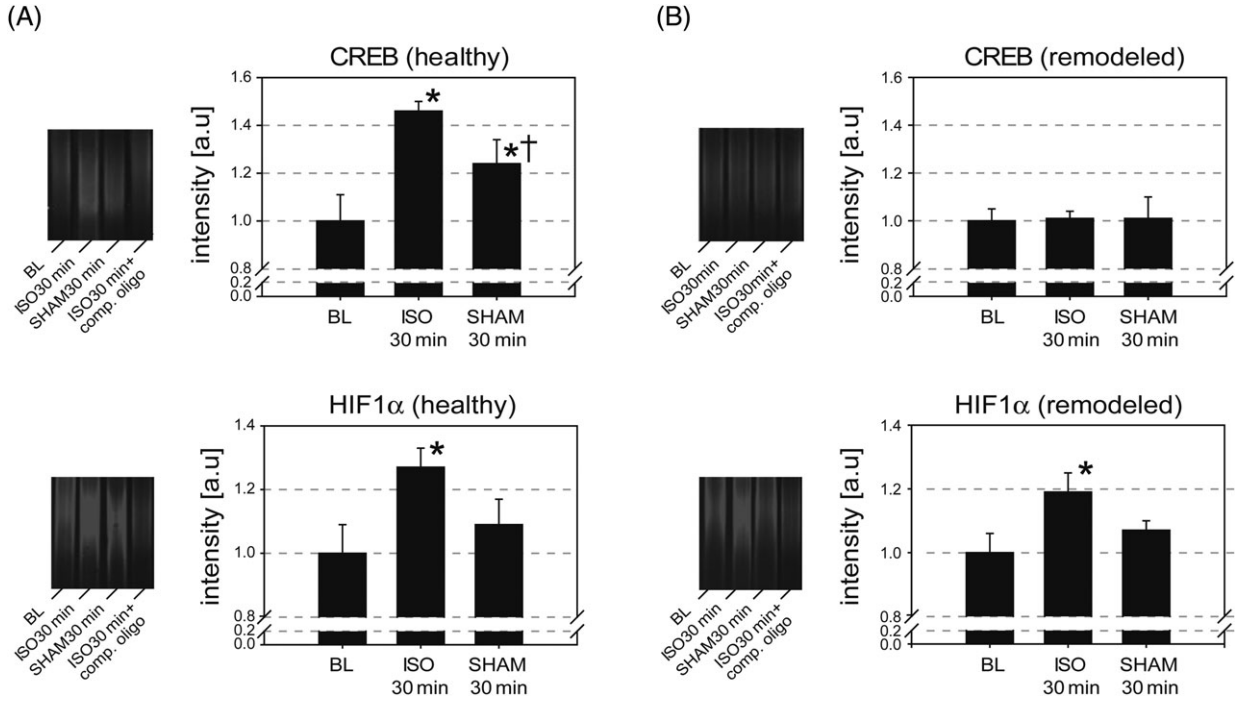

Figure 6 Electrophoretic mobility shift assays of HIF1α and CREB in healthy (*A*) and remodelled (*B*) hearts (gels with corresponding densitometry). ISO30 min: nuclear extracts from hearts exposed to isoflurane preconditioning harvested 30 min after termination of inhalation (2.1 vol% for 90 min). SHAM30 min: nuclear extracts from respective sham group (oxygen alone). Gels show additional lines with ISO30 min plus 200-fold excess of unlabelled consensus competitor oligonucleotide (comp. oligo) confirming specificity of the detected fluorescence signal for HIF1α and CREB, respectively. Data are mean $\pm$ SD ($n = 4$ per group). *$P < 0.05$ vs. baseline (BL) and healthy hearts, respectively. $^{\dagger}P < 0.05$ vs. ISO30 min.

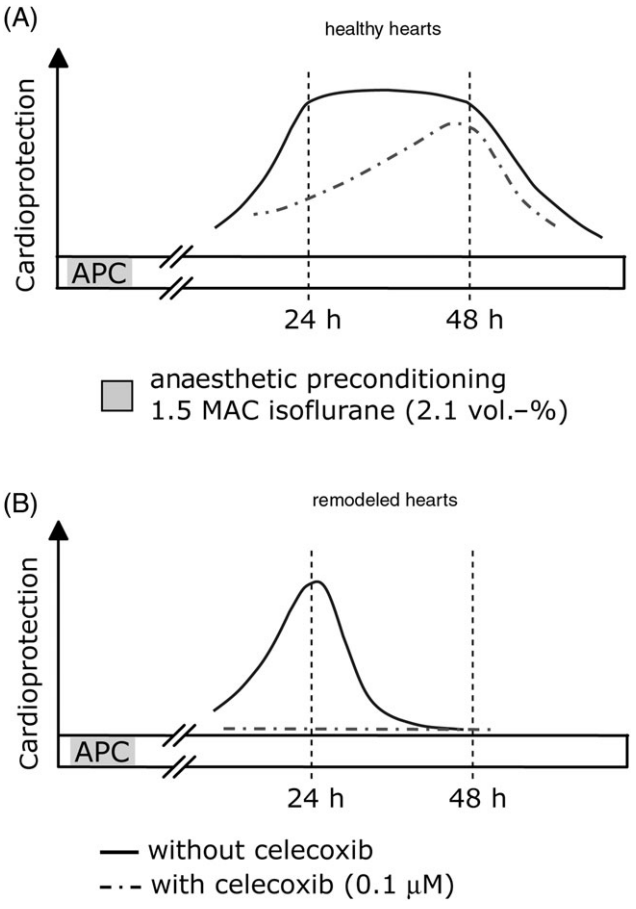

Figure 7 'Second window of protection' in healthy and remodelled hearts. The panels show the second window of protection at 24 and 48 h after isoflurane exposure (APC) in healthy (*A*) and infarct remodelled (*B*) hearts in the absence (solid line) and presence (dashed line) of celecoxib (0.1 μM).

of the heart to stress.[23] In contrast to early preconditioning, delayed preconditioning has strong and more reliable anti-stunning and anti-infarct effects making it more clinically relevant. It causes an increase in prostaglandin biosynthesis in cardiac tissue via the enhanced expression of COX-2 catalysing the conversion of arachidonic acid to prostanoids.[24] COX-2 is constitutively expressed in the heart at lower levels[25] but rapidly induced through the activation of several transcription factors.[3,23] Prostanoids such as prostacyclin (PGI_2) open mitochondrial K_{ATP} channels,[26] key players in isoflurane-induced cardioprotection.[27] Inhibition of the rate-limiting enzyme COX-2 abolishes the infarct-sparing effect of delayed preconditioning,[24] but also the protection by early preconditioning.[28] Using healthy animal models from different species, previous studies reported on late cardioprotection after exposure to volatile anaesthetics,[29,30] and identified reactive oxygen and nitrogen species released by nitric oxide synthase as triggers,[31] inducible nitric oxide synthase,[32] 12-lipoxygenase,[8] and endothelial nitric oxide synthase[33] as mediators, and sarcolemmal and mitochondrial K_{ATP} channels as end effectors[6] of delayed anaesthetic preconditioning. Hitherto, however, only one study with healthy rabbit hearts investigated the role of COX-2 in isoflurane-induced delayed preconditioning using the COX-2 inhibitor celecoxib.[7] Our study now confirms COX-2 as an obligatory mediator in isoflurane-induced delayed preconditioning of healthy rat hearts, and further extends its critical role in cardioprotection to diseased post-infarct remodelled myocardium.

Structural changes due to loss of viable tissue and scar formation,[12] alterations in metabolism,[13] and cellular signalling[14,34,35] put the remodelled myocardium at risk for further ischaemic damage. Experiments from muscle slices of right atrial appendices of patients with failing hearts indicate that remodelled myocardium is less amenable to innate protection by preconditioning.[36] At the molecular level, a gene program resembling the foetal program is activated and protective signalling pathways were reported to be lost in various disease models associated with remodelling.[37] Complete refractoriness to early ischaemic preconditioning as opposed to pharmacologic preconditioning by diazoxide, was reported in a rabbit infarct model, for which interruption of signal transduction between G-protein coupled receptors and protein kinase C was found to be responsible.[38] Conversely, erythropoietin-mediated preconditioning was preserved in post-infarct remodelled rat myocardium despite the disruption of the erythropoietin receptor–PI3K–PKB pathway via up-regulation of the suppressor of cytokine signalling protein-1.[39] In this case, compensatory PI3K-independent activation of ERK upstream of the guanylyl cyclase-mitochondrial K_{ATP} channel pathway restored the protective phenotype. To date, no data is available with respect to delayed protection after pharmacologic or ischaemic preconditioning in post-infarct remodelled hearts. In contrast to our previous findings on early isoflurane-induced preconditioning[15] and postconditioning in remodelled myocardium,[16] where protection against ischaemia-reperfusion was fully preserved, our current study on delayed isoflurane preconditioning clearly indicates that ventricular remodelling alters protective signalling rendering the heart more vulnerable to ischaemia-reperfusion injury. In search of mechanisms for this remarkable vulnerability, we tested a number of COX-2-inducing transcription factors to see whether their nuclear translocation would be impaired in the diseased state. Increased HIF1α protein levels were previously reported after isoflurane exposure in rat myocardium[40] and were similarly regulated in the current study in healthy and diseased hearts. HIF1α is an oxygen-dependent transcription factor activating an array of genes required for the adaptation to hypoxia, including COX-2. In contrast, CREB was exclusively mobilized in healthy but not remodelled hearts, which showed increased expression of the transcriptional repressor and CREB-antagonist ICER.[41] ICER represses transcription either by heterodimerization with activating forms of CREB and other bZIP domain-containing transcription factors, or by competing with these proteins for DNA binding.[42] Due to the homology between ICER and CREB, we cannot completely rule out that both CREB and ICER bind to the CRE-consensus oligos used in our experiments. However, the DNA-binding activity is lower in remodelled hearts than in healthy hearts indicating that ICER may not or only weakly bind to CREB-oligos (data not shown). The results of our experiments raise the possibility that the observed lack of CREB-DNA binding in the remodelled hearts might be due to heterodimerization of CREB with other transcriptional factors including ICER that thereby could antagonize CREB. This hypothesis should be proven in future experiments.

COX-2 inhibitors increase the number of cardiovascular complications specifically in patients with preexisting heart disease.[43] A recent pig study impressively showed that peri-infarct inhibition of COX-2 by celecoxib decreased myocardial function and increased left ventricular remodelling and mortality.[44] In the current study, we also evaluated the effects of low and higher concentrations of the clinically used celecoxib on late isoflurane protection. The IC_{50} of celecoxib for COX-2 (0.04 μM) is 375-times lower than for cyclooxygenase-1,[45] but celecoxib has additional unspecific inhibitory effects on other key enzymes.[21] A single oral administration of 200 mg celecoxib results in a plasma concentration of 700–1100 μg/L, whereby only a small amount (3%) of the drug is unbound and biologically active.[45] Hence, the effective tissue concentration under clinical conditions is close to 0.1 μM, as used in our study in the protein-free buffer perfusing the isolated hearts. Higher concentrations of unbound drug in the range of 1 μM are unusual, but were reported in patients with CYP2C9 deficiency due to genetic polymorphisms.[45] Our results clearly show that even low concentrations of celecoxib suffice to abolish delayed protection by isoflurane. Increased expression of ICER was previously reported to downregulate prosurvival Bcl-2,[41,46] and thus might well be responsible for the higher susceptibility of infarct hearts to celecoxib. From a translational point of view, our data suggest that the use of selective COX-2 inhibitors should be minimized in at-risk patients with significant ventricular remodelling. Finally, our results imply that diseased myocardium requires more frequent intermittent preconditioning stimuli to maintain the protected state.

Although previous studies showed the significance of CREB in establishing delayed preconditioning,[47] our experiments cannot exclude that other factors contributed to the shorter duration of COX-2 upregulation. Our study focused on COX-2 in healthy and diseased rat hearts, and did not investigate molecular cross-talk between iNOS and COX-2 or other kinases such as protein kinase B. Since ischaemic postconditioning was recently reported to enhance delayed preconditioning by further up-regulation of COX-2,[48] future studies should test whether ischaemic and pharmacologic postconditioning could fully restore delayed protection by preconditioning in post-infarct hearts. Some previous studies showed divergent mechanisms during the early, late and final stage of delayed preconditioning.[49] Although our findings suggest that delayed isoflurane preconditioning is mediated via the same mechanisms throughout its duration, we cannot exclude that other or additional mechanisms may be involved in the final stage (at 72 h) of late isoflurane protection. Finally, timing of protection is often species-dependent and may be different in humans.

In summary, our study shows that post-infarct remodelling in rat hearts hinders sustained COX-2 expression and activity after isoflurane preconditioning and thus narrows the second window of protection. Isoflurane induces nuclear translocation of HIF1α in both healthy and remodelled hearts, but fails to mobilize the transcription factor CREB in diseased ICER-overexpressing hearts. The study further demonstrates that innate protection of remodelled myocardium is exceptionally vulnerable to COX-2 inhibition. Hence, protection by delayed isoflurane preconditioning varies with the disease state of the heart and concomitant medication.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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Molecular Evidence of Late Preconditioning After Sevoflurane Inhalation in Healthy Volunteers

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BACKGROUND: Late preconditioning by volatile anesthetics evolves in response to transcriptional changes. We hypothesized that sevoflurane inhalation would modify the transcriptome in human blood and modulate the expression of adhesion molecules in white blood cells consistent with the occurrence of a late preconditioning phase.

METHODS: Five healthy male subjects inhaled sevoflurane at an end-tidal concentration of 0.5%–1.0% for 60 min. Venous blood samples were collected at baseline, after 15 and 60 min of inhalation, and 6, 24, 48, and 72 h thereafter and immediately processed for flow cytometry and mRNA extraction and hybridization to Affymetrix U133 Plus 2.0 microarrays. Data were analyzed using Significance Analysis of Microarray and Gene Set Enrichment Analysis and confirmed by real-time reverse transcription polymerase chain reaction. L-selectin (CD62L) and β_2-integrin (CD11b) expression was determined on granulocytes and monocytes using flow cytometry.

RESULTS: Sevoflurane inhalation rapidly and markedly altered gene expression in white blood cells. Key transcripts potentially involved in late preconditioning or organ protection including paraoxonase, 12-lipoxygenase, heat shock protein 40, chemokine ligand 5, and phosphodiesterase 5A were regulated in response to sevoflurane. Sevoflurane further decreased transcripts involved in peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) signaling and fatty acid oxidation. Reduced L-selectin (CD62L) expression on granulocytes accompanied with increased resistance to inflammatory activation was present at 24 to 48 h after sevoflurane exposure.

CONCLUSIONS: Sevoflurane at subanesthetic concentrations modifies blood transcriptome and decreases the expression of the proinflammatory L-selectin (CD62L), consistent with a "second window of protection" in humans.

(Anesth Analg 2007;105:629–40)

Preconditioning is a biological process observed in multiple organs, whereby a transient stressful stimulus induces a protective state against a more prolonged, potentially lethal insult. It can be induced by brief ischemic episodes or by drugs such as volatile anesthetics (1,2). This protection has two phases: an early phase, immediately operating after the application of the preconditioning stimulus and lasting for 2–3 h, and a late phase, evident after 12–24 h but lasting for up to 3 days. Although early preconditioning is predominantly based on multiple, fast-acting intracellular phosphorylation signaling steps (3), the second window is a result of transient altered gene activity (4) and depends on novel protein expression (5). Volatile anesthetic-induced preconditioning was reported to be effective in various cell types, including cardiac myocytes (6,7), and endothelial and smooth muscle cells (8). In addition, several experimental studies provide evidence of a "second window of protection" elicited by volatile anesthetics in mouse (9), rat (10–12), and rabbit hearts (13–15). In contrast, one study with isoflurane was unable to show delayed cardioprotection in an *in vivo* dog model (16). However, it is unknown whether late preconditioning elicited by volatile anesthetics also occurs in humans.

Recruitment of inflammatory cells to sites of ischemic injury contributes significantly to organ dysfunction. Focal accumulation of leukocytes is mediated by the interaction of selectins with their endothelial counterligands, while firm attachment of leukocytes and transmigration requires activation of β_2-integrin (CD11b) (17). Using a highly controlled human *in vivo*

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Reprints will not be available from the author.

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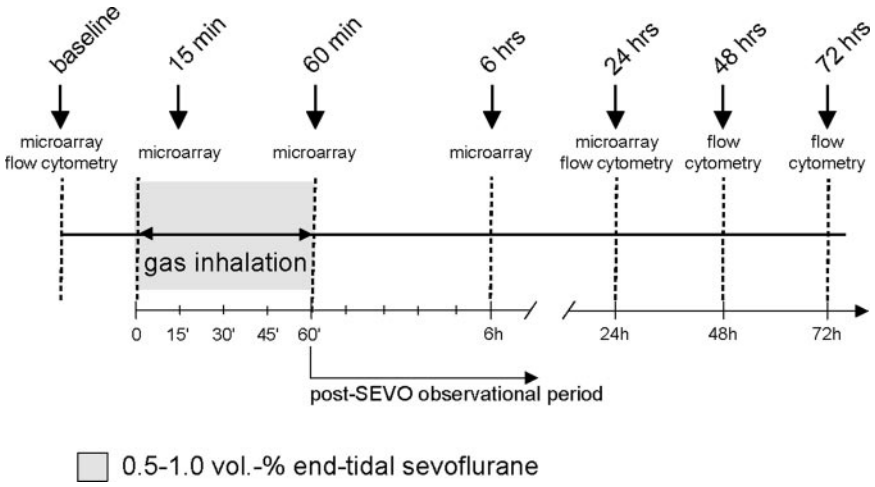

Figure 1. Study protocol. Sevoflurane (SEVO) was inhaled for 60 min at 0.5%–1.0% end-tidal concentration. Blood samples were collected at indicated time points and used for microarray analysis and flow cytometry. Separate experiments with inhalation of 50% oxygen without sevoflurane served as control.

model of endothelial dysfunction, we have recently shown that sevoflurane inhalation at subanesthetic concentrations decreases activation of β_2-integrin (CD11b) on granulocytes and monocytes after ischemia-reperfusion of the forearm in healthy volunteers, consistent with an early preconditioning of the endothelium (18). We next wanted to know whether sevoflurane inhalation would also regulate gene and protein expression in the blood in accordance with the occurrence of a late window of anesthetic-induced preconditioning in humans. Specifically, we hypothesized that sevoflurane inhalation would modulate blood transcripts potentially involved in late protection and reduce the expression of L-selectin (CD62L) and β_2-integrin (CD11b) 24–48 h later in humans.

METHODS

Study Subjects

In this study, five healthy male volunteers with Caucasian genetic background, aged 32 ± 5 (27–39) yr and with a body mass index of 23 ± 6 (21–28) kg/m^2 gave informed signed consent. All participants were nonsmokers and refrained from caffeine and dark chocolate containing endothelium-protective flavonoids from 24 h before until 72 h after sevoflurane inhalation. The subjects fasted overnight, and were pretreated with a single dose of oral ranitidine (300 mg) the evening before sevoflurane inhalation. The research project was performed in accordance with the Declaration of Helsinki (2000), and was approved by the local ethics committee of the University Hospital Zurich, Switzerland.

Study Protocol

Figure 1 shows the time course of the experiments. After inserting a cannula into a cubital vein and the administration of 500 mL Ringer solution, the subjects inhaled sevoflurane in 50% oxygen to achieve a subanesthetic end-tidal concentration of between 0.5% and 1.0% for 60 min. Sevoflurane was inhaled by the spontaneously breathing volunteers using a facemask connected to the common gas outlet of an anesthesia machine (Siemens Servo 900D ventilator, Siemens Life Support Systems, Sona, Sweden), as previously described (18). Blood samples were taken from the cubital vein (opposite side of blood pressure cuff) before gas inhalation (baseline), after 15 and 60 min of sevoflurane administration, and 6, 24, 48, and 72 h thereafter. One week after sevoflurane inhalation, separate experiments, conducted in a crossover mode with the same individuals, served to clarify whether inhalation of 50% oxygen without sevoflurane and administration of a single oral dose of ranitidine (300 mg) would affect gene expression. Monitoring consisted of intermittent noninvasive arterial blood pressure measurements, 5-lead electrocardiogram, end-tidal CO_2, end-tidal sevoflurane concentrations (Hellige VICOM-SM SMU 612; PPG, Freiburg, Germany), and Bispectral Index (A2000 monitor® with three adhesive electrodes to the forehead, single channel: Fp1–Fpz, version 3.3; Aspect Medical Systems, Natick, Massachusetts) for depth of sedation.

Transcriptional Profiling of Human Blood Samples

Total RNA was extracted from whole blood samples (collected in tripotassium-EDTA tubes) after centrifugation using RiboPure™-Blood Kit (Ambion, Huntington, United Kingdom). Microarray analysis was performed following the "minimum information about a microarray experiment" guidelines (19). Briefly, the quality of the isolated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Delaware) (260/280 nm ratio between 1.8 and 2.1 and 28S/18S ratio within 1.5–2). Probe labeling and purification was achieved as previously described (20). From four of the five subjects, samples collected at baseline, 15 min, 1, 6, and 24 h were processed and hybridized to an individual Affymetrix GeneChip® Human Genome U133 Plus 2.0 arrays (total of 20 arrays) for 16 h at 45°C. Arrays were washed using an Affymetrix Fluidics Station and scanned using an Affymetrix GeneChip Scanner 3000 at a resolution of 3 μm. Normalization and computation of expression values were performed using the robust multichip average method (21). From the global gene expression

matrix, which contains the expression values of the 54′675 probe sets at all time points, the expression values at baseline and after 1 h of sevoflurane inhalation (peak transcriptional change) were selected and used to determine: 1) differentially expressed genes using the Significance Analysis of Microarrays (SAM) algorithm (22) and 2) differentially expressed pathways using Gene Set Enrichment Analysis (GSEA) 23, as previously described in detail (20,24). In SAM, each gene is assigned a score on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene. Genes with scores more than a threshold are deemed potentially significant and the algorithm provides an estimate of the false discovery rate (FDR), i.e., the expected proportion of false positives among the transcripts called significant. Conveniently, GSEA aggregates the per gene statistics across genes within a gene set, thus making it possible to detect situations where the genes in a predefined set change in a small but coordinated way. GSEA calculates an enrichment score (NES) for a given gene set using a ranked list of all genes and infers statistical significance (expressed as a P value and a FDR) of each NES against NES background distribution calculated by permutation of the original data set. A cut-off median FDR of 3% was used in SAM analysis, and $P < 0.05$ was used in GSEA analysis to obtain the ranked lists of differentially expressed genes and pathways, respectively. To define a standardized measure of gene expression over time (the mean centroid), we normalized the gene expression levels of the top up- and down-regulated genes to a mean of 0 and a variance of 1 across all 20 samples over time. Microarray analysis was performed for four individuals only to decrease related costs. All other analyses were performed for all five study subjects.

Transcript Validation Using Real-Time Reverse Transcriptase-Polymerase Chain Reaction

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed for four randomly selected genes to confirm microarray data (please see Supplementary Table S1 available at www.anesthesia-analgesia.org), as previously described (20,25). First strand cDNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen, Basel, Switzerland) and oligo-dT as primer. RT-PCR quantification of the selected genes was performed on a Stratagene MX3000 real-time sequence detector instrument (Stratagene Europe, Amsterdam, The Netherlands) using the Brilliant SYBR green QPCR Master Mix (Stratagene Europe). Amplification reactions were conducted with an initial step at 90°C for 3 min followed by 20–35 cycles. All PCR reactions were performed in triplicates, and ribosomal 18S (a constitutively expressed gene) was used as reference control. Predicted size of PCR products was confirmed by agarose gel electrophoresis.

Determination of L-Selectin (CD62L) and β_2-Integrin (CD11b) Expression in the Blood Using Flow Cytometry

L-selectin (CD62L) is an early indicator of neutrophil activation, while β_2-integrin (CD11b) expression is a later event of the leukocyte-endothelial interaction. Collected heparinized blood samples were immediately processed for flow cytometry. The expression of the adhesion molecules was determined at baseline, 24, 48, and 72 h after sevoflurane exposure in all five volunteers. Two microliters of 0.5 mM *N*-formyl-methionyl-leucyl-phenylalanin (fMLP) was added to 1 mL of collected whole blood (1 μM final fMLP concentration) and incubated for 10 min at room temperature. Unstimulated blood served as control. Subsequently, 3 μL of the primary fluorochrome-labeled antibody was added to 50 μL of blood in endotoxin-free tubes and incubated in the dark for 10 min at room temperature. Lysis buffer (450 μL) (Becton Dickinson, Basel, Switzerland) was added and incubated for an additional 20 min at room temperature. The lysates were fixed for 30 min in 0.5 mL 0.2% paraformaldehyde solution at room temperature. The samples were centrifuged, and the cell pellets were suspended in 0.5 mL of TLR buffer, and stored at 4°C in the dark. The FACSCalibur (Becton Dickinson) flow cytometer was used to measure R-phycoerythrin (PE)-fluorescence at 580 nm and FITC-fluorescence at 515 nm. White blood cells were distinguished from each other by typical physical characteristics, resulting in well-delineated cellular subpopulations that are easily identified on forward and side-scatter plots. Monoclonal antibodies for polymorphnuclear granulocytes (CD15, PE-labeled, clone 80H5, Immunotech, Marseille, France) and monocytes (CD14, FITC-labeled, clone 61D3, eBioscience, Wembley, United Kingdom) further served for identification of cellular subgroups. CD62L and CD11b expression were measured by fluorescence intensity of PE-conjugated monoclonal antibodies directed against CD62L (FITC-labeled, clone DREG56, eBioscience) and CD11b (PE-labeled, clone 2LPM19C, DAKO, Glostrup, Denmark). Results were compared to isotype-matched control IgG (PE-labeled IgG, eBioscience and FITC-labeled IgG, Becton Dickinson). A minimum of 20,000 events was counted on each sample.

Statistical Analysis

Data are given as mean (SD). Repeated-measures ANOVA were used for comparison. Student-Newman-Keuls test was used for *post hoc* analysis. $P < 0.05$ was considered significant. Analyses were performed using StatView Version 5 (SAS Institute, Chicago, IL).

RESULTS

The 60 min period of sevoflurane inhalation at sedative concentrations (0.5%–1%) had only marginal effects on arterial blood pressure and end-tidal CO_2

Table 1. Recorded Parameters at Baseline and During Inhalation of Small-Dose Sevoflurane

	Subject ID	V_1[a,b]	V_2[a,b]	V_3[a,b]	V_4[a,b]	V_5[b]
Before SEVO	SBP (mm Hg)	110	112	124	111	113
	HR (bpm)	72	47	50	61	65
	Spo_2 (%)	100	100	100	100	100
	BIS	95	95	98	97	98
During SEVO	ET (SEVO)[c] (vol %)	1.0	0.70	0.95	0.82	1.0
	ET (Co_2)[c] (vol %)	3.8	5.0	4.5	4.4	4.2
	SBP[d] (mm Hg)	95	108	107	100	103
	HR[d] (bpm)	57	46	52	72	55
	Spo_2[c] (%)	99	100	98	100	100
	BIS[c]	68	69	82	79	72

SEVO = sevoflurane; SBP = systolic blood pressure; HR = heart rate (in beats per minute or bpm); Spo_2 = oxygen saturation (finger tip); BIS = Bispectral Index; ET(SEVO) = end-tidal sevoflurane concentration; ET(Co_2) = end-tidal Co_2.

[a] The blood samples of these subjects were used for gene chip analysis (N = 4).

[b] The blood samples of these subjects were used for flow cytometry (N = 5).

[c] Mean values during sevoflurane inhalation.

[d] Values recorded after 30 min of sevoflurane in 50% oxygen inhalation.

concentrations (Table 1). The participants were responsive to verbal commands or tactile stimuli at all times. The Bispectral Index ranged between 68 and 82 on average. All subjects tolerated the procedure without complications.

Sevoflurane Inhalation at Subanesthetic Concentrations Markedly Altered Gene Expression in the Blood of Healthy Volunteers

We first tested whether sevoflurane at subanesthetic concentrations is capable of modulating gene expression in humans. For this purpose, mRNA from whole blood was isolated at different time points after sevoflurane application and used for gene chip hybridization. Gene chip data were independently confirmed with RT-PCR, which showed the same results (please see Supplementary Table S2 available at www.anesthesia-analgesia.org). Significance Analysis of Microarrays (SAM) revealed 74 upregulated (changes in expression more than 35% and median FDR <3%) and 63 down-regulated transcripts (median FDR <3%) after 60 min of sevoflurane exposure (for complete list of transcripts, please see Supplementary Table S3 available at www.anesthesia-analgesia.org). Three volunteers exhibited peak gene regulations after 1 h, while one subject exhibited peak transcriptional responses after 15 min of inhalation (Fig. 2). Using microarrays covering the entire human genome (>54,000 probes for the approximately 30,000 human genes), our data confirm the profound effects of volatile anesthetics on the transcriptome, as previously reported in rat hearts (24,25), and further extend these findings to human blood in response to subanesthetic concentrations of sevoflurane.

Our analysis detected a number of up- and down-regulated transcripts already known to be involved in the protection of the heart or other vital organs (Fig. 3; please see Supplementary Table S3 available at www.anesthesia-analgesia.org). In particular, these included the up-regulated transcripts paraoxonase, 12-lipoxygenase, DnaJ (Hsp40), and the down-regulated transcripts chemokine ligand 5 (a potent neutrophil chemoattractant, also called epithelial neutrophil activating protein-78) and phosphodiesterase 5A. Control experiments with inhalation of 50% oxygen for 1 h did not show any transcriptional regulation of paraoxonase, 12-lipoxygenase, and acyl-CoA synthase (Fig. 4). Sevoflurane inhalation down-regulated transcripts involved in peroxisome proliferator-activated receptor (PPAR) regulation and fatty acid oxidation in blood cells. We have recently shown that sevoflurane anesthesia down-regulates PPARα and its coactivator protein, PGC-1α, which transcriptionally regulate metabolism and lead to a shift in fuel preference away from fatty acid oxidation in the heart (20). In the present study, GSEA confirmed the coordinate down-regulation of genes involved in fatty acid oxidation after sevoflurane inhalation (NES = -0.55, P = 0.00, FDR q-value = 0.08) in blood cells, which closely correlated with PPAR-related genes (PGC-1α pathway: NES = -1.22, P = 0.02, FDR q-value = 0.43) (Figs. 5A–C). Finally, a large number of regulated transcripts emerged with yet unknown function in the context of organ protection (please see Supplementary Table S3 available at www.anesthesia-analgesia.org).

Sevoflurane Inhalation Reduces L-Selectin (CD62L) Expression on Leukocytes and Induces Cellular Resistance to Inflammatory Stimulation 24–48 h After Exposure in Humans

To elucidate whether sevoflurane inhalation modulates protein expression consistent with a "second window" of preconditioning, we determined the expression of the important adherence molecules β_2-integrin (CD11b) and L-selectin (CD62L) from cubital blood collected up to 72 h after sevoflurane application. Expression levels were determined separately on granulocytes (CD14 positive) and monocytes (CD15 positive). Sevoflurane administration significantly reduced (approximately 25%) the expression of L-selectin (CD62L) in granulocytes (P = 0.004), but had only a marginal effect on monocytes (Figs. 6A and B). These changes were most evident at 24 and 48 h after inhalation. In contrast, there was no decrease in

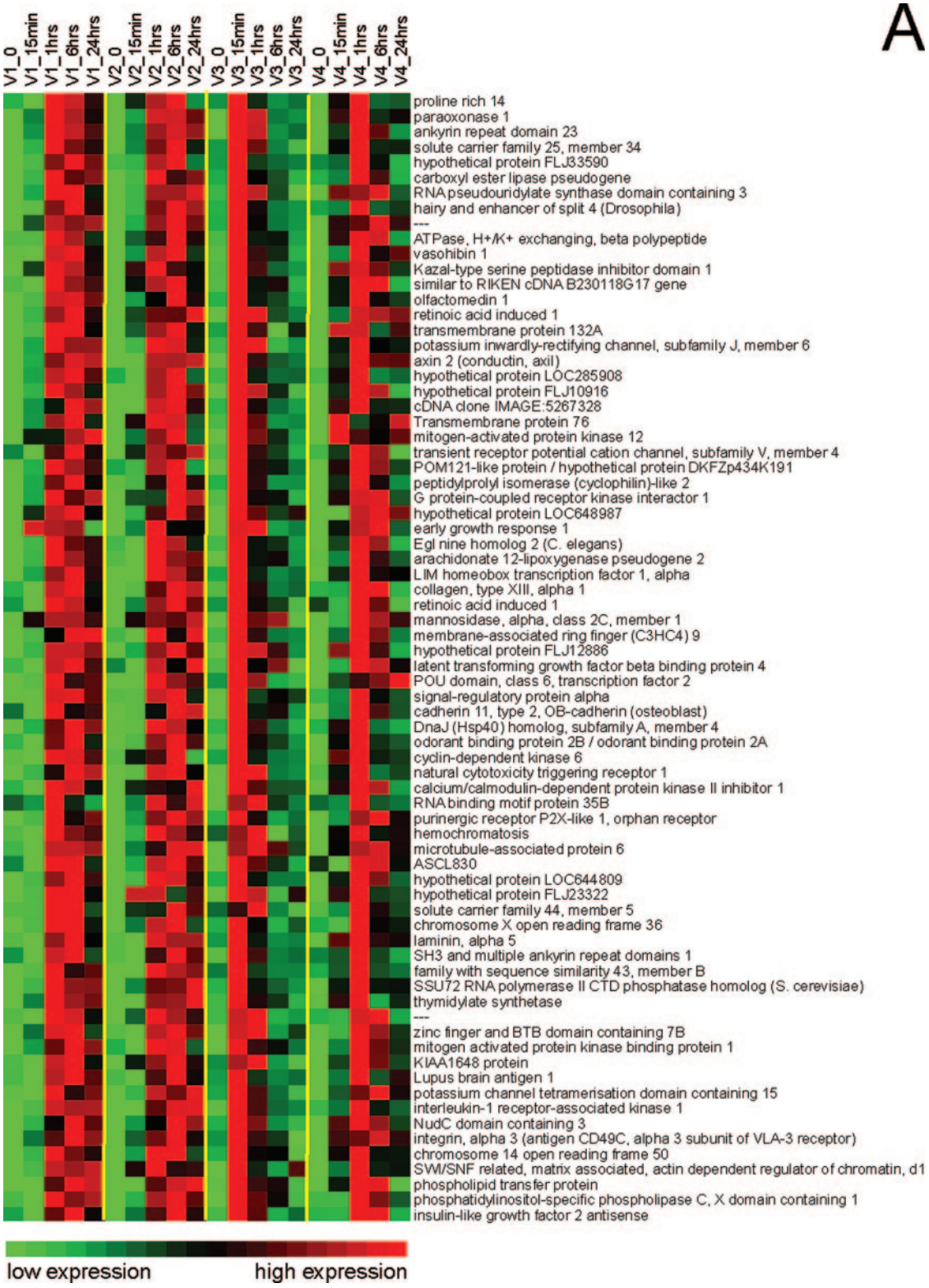

Figure 2. Time course of relative changes in blood transcriptome in response to sevoflurane inhalation. Significance Analysis of Microarrays identified a total of 137 transcripts, 74 up-regulated >1.35-fold (Panel A) and 63 down-regulated (Panel B), that were differentially expressed after 1 h of sevoflurane inhalation as compared to baseline. Expression levels are depicted as color scale from red (high expression) to green (low expression). Each numbered square indicates the expression value of a transcript (rows) in a specific subject (columns) labeled with V1 to V4 (n = 4; please see Supplementary Table S2 available at www.anesthesia-analgesia.org for more details).

β_2-integrin (CD11b) expression in monocytes and granulocytes (data not shown). Importantly, granulocytes stimulated with the bacterial oligopeptide fMLP exhibited markedly reduced shedding of L-selectin (CD62L) (Fig. 6C and D, P = 0.009), indicating resistance to inflammatory stimulation. L-selectin (CD62L)

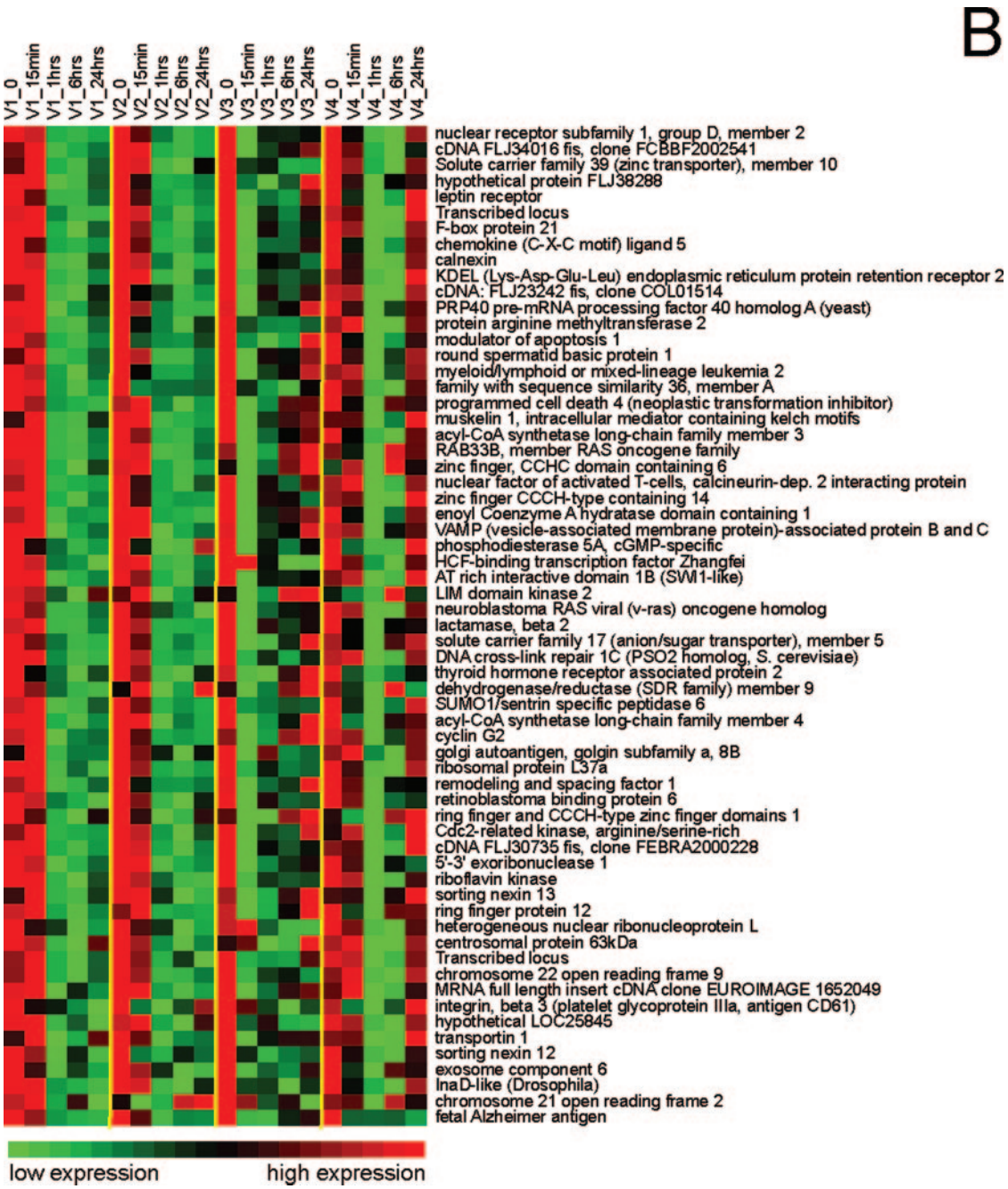

Figure 2. (Continued).

shedding was maximally inhibited 24 to 48 h after sevoflurane exposure.

DISCUSSION

The salient findings of the present study are as follows. First, sevoflurane inhalation, even at low subanesthetic concentrations, rapidly altered the blood transcriptome on a genome-wide scale in healthy subjects, a prerequisite for late preconditioning, which depends on *de novo* protein synthesis. The observed transcriptional changes specifically involved genes with known biological significance in the context of late preconditioning or organ protection. In accordance with our previous findings in human hearts exposed to sevoflurane (20), genes involved in fatty acid oxidation, regulated by the PCG1α-pathway, were similarly down-regulated in the blood. Second, 24 to 48 h after sevoflurane exposure, i.e., consistent with the occurrence of a late or second window of preconditioning, the expression of L-selectin (CD62L), a key inflammatory adhesion molecule responsible for the tethering of leukocytes to the endothelium (17), was reduced by approximately 25% on granulocytes, which further exhibited an increased resistance to inflammatory stimulation. Taken together, using blood as a model system and standardized experimental conditions, we provide for the first time molecular evidence that the anesthetic gas, sevoflurane, can induce late preconditioning in humans. Clearly, our

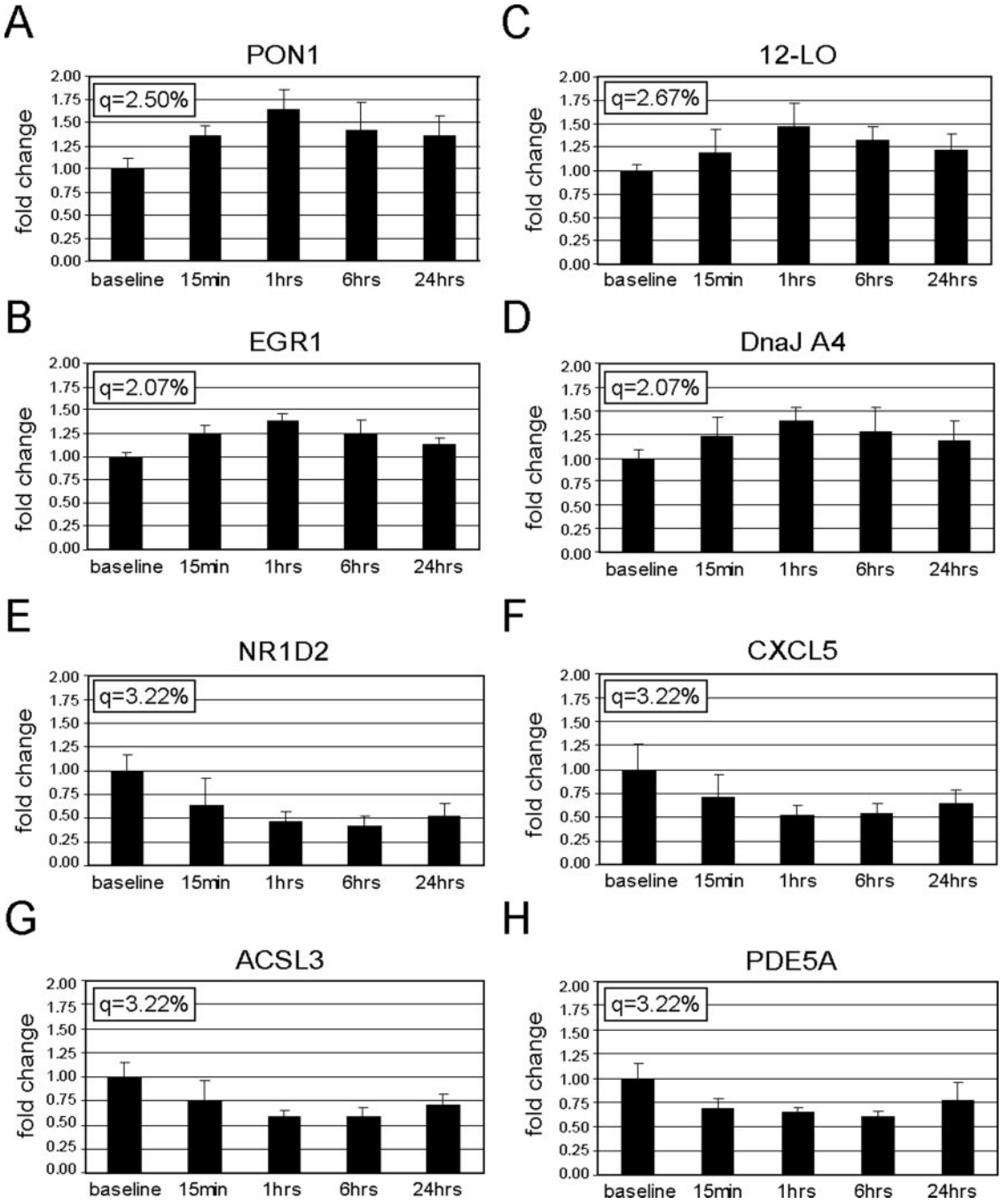

Figure 3. Microarray expression levels of significantly regulated transcripts potentially involved in late preconditioning and organ protection after sevoflurane inhalation (Panel A–H). Individual q-values, as obtained by Significance Analysis of Microarrays, are given for each transcript. PON1 = paraoxonase 1, 12-LO = 12-lipoxygenase, EGR1 = early growth response 1, DnaJA4 = heat shock protein 40, member A4, NR1D2 = nuclear receptor family 1D, member 2, CXCL5 = chemokine ligand 5, ACSL3 = acyl-CoA synthase, long-chain 3, PDE 5A = phosphodiesterase 5A. Data represent mean ± SD (n = 4).

findings should be confirmed in the clinical setting with patients.

Animal Models of Late Preconditioning by Volatile Anesthetics

The potential of late preconditioning to provide enduring protection (lasting 30-fold longer compared to early preconditioning) is of potential clinical importance. However, it was elusive whether late preconditioning, i.e., protection developing 24 to 48 h after the application of the preconditioning stimulus, would also occur in humans in response to volatile anesthetics. Animal studies indicate that volatile anesthetics offer protection from ischemia-reperfusion injury in heart (12) and brain (26) tissue lasting for up to 48 h. Tonkovic-Capin et al. (13) first reported successful late preconditioning of the heart in a rabbit model using isoflurane at 1% end-tidal concentration. Subsequent studies confirmed these findings in rats (10–12) and mice (9). Interestingly, emulsified formulations of volatile anesthetics equivalent to only 0.2 MAC, and

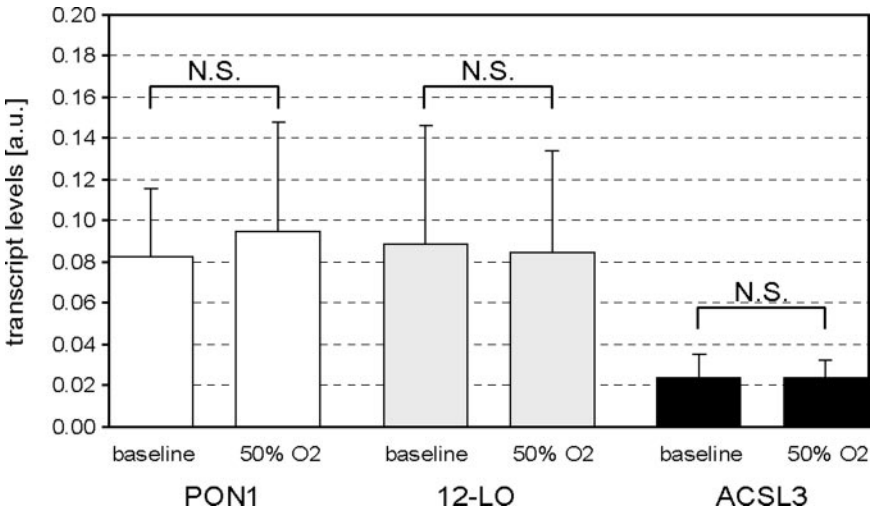

Figure 4. The levels of selected transcripts (paraoxonase 1, PON1; 12-lipoxygenase pseudogene, 12-LO; acyl-CoA synthase, long-chain 3, ACSL3) before and after inhalation of 50% O_2 for 1 h (for primers, please see Supplementary Table S1 available at www.anesthesia-analgesia.org). Data represent mean ± SD ($n = 4$).

with no apparent sedation markedly reduced infarct size in rabbit hearts when administered IV 24 h before test ischemia (15). Signaling pathways involved in volatile anesthetic-induced late preconditioning are reminiscent of those observed in other types of late preconditioning (4,5), including reactive oxygen and nitrogen species as triggers, and 12-lipoxygenase, cycloxygenase-2, and inducible nitric oxide synthase (iNOS) as mediators or effectors, and the magnitude of the afforded protection is similar. In accordance with these animal studies, the present study now provides, for the first time, evidence of a late protective phenotype after sevoflurane inhalation in humans.

Genetic Reprogramming by Sevoflurane: A Prerequisite for Late Preconditioning

Late preconditioning directly depends on transient transcriptional or translational changes (for h), and the delayed and prolonged protection is consistent with altered protein expression (for days). To test our hypothesis, we used the easily accessible blood compartment as a human model system, and determined the time course of transcriptional changes after sevoflurane inhalation in healthy volunteers. No transcriptional changes were measured after inhalation of 50% oxygen alone. However, genes encoding key protective proteins were significantly regulated after sevoflurane inhalation within only a short period, consistent with results obtained in humans after remote ischemic preconditioning of the limb (27). The observed changes in gene expression were only transient. Peak transcriptional changes occurred within 1 h of sevoflurane inhalation, but most of the changes disappeared after 24 h. This time course is characteristic for the transcriptional changes underlying the second window of protection in the heart. Salloum et al. reported peak iNOS mRNA expression in mice already 1–2 h after a single dose of sildenafil, a late preconditioning-inducing drug (4). Subsequently, the increase in iNOS mRNA completely vanished, but changes in cardioprotective iNOS protein expression were not measured before 24 h after the application of the triggering drug (4). This contention is consistent with our findings of an early transient transcriptional response (within hours) followed by late more persistent changes in protein expression (24–48 h later). Of note, changes in mRNA and protein expression were only modest (30%–40% compared to baseline) similar to our human study, but had marked effects on infarct size (from 30% to 6% of the area at risk). Interestingly, 12-lipoxygenase, which was recently implicated in delayed cardiac protection in mice (9), was upregulated. Conversely, phosphodiesterase 5A was down-regulated by sevoflurane. Inhibition of phosphodiesterase 5A by sildenafil was previously shown to induce late preconditioning in mice through the iNOS pathway (4). Additional potentially protective transcripts that were regulated in the blood transcriptome included the antioxidant paraoxonase (28), heat shock protein 40, known to prevent cytochrome c release from mitochondria, and a number of transcripts with less well-established function in the context of late preconditioning, such as EGR1, a master switch coordinating upregulation of stress genes, or NR1D2, a nuclear hormone receptor acting as a link between metabolism and inflammation (29). Collectively, our data show that sevoflurane inhalation reprograms blood transcriptome toward a defensive phenotype in humans.

Sevoflurane-Induced Delayed Protection Against Leukocyte-Endothelial Activation

Leukocytes have a central role in the pathogenesis of ischemia-reperfusion damage. In the present study, we show that inhalation of sevoflurane decreases the expression of L-selectin (CD62L) in granulocytes and attenuates their responsiveness to inflammatory stimulation 24 to 48 h after exposure, consistent with a second window of protection. Extravasation of leukocytes is mediated by adhesion molecules, and exacerbates tissue injury after restoration of blood supply to the site of ischemia (30,31). In the dynamic interaction between leukocytes and endothelium, the cell surface receptor L-selectin (CD62L) and its proteolytic shedding mediate the initial steps of leukocyte tethering

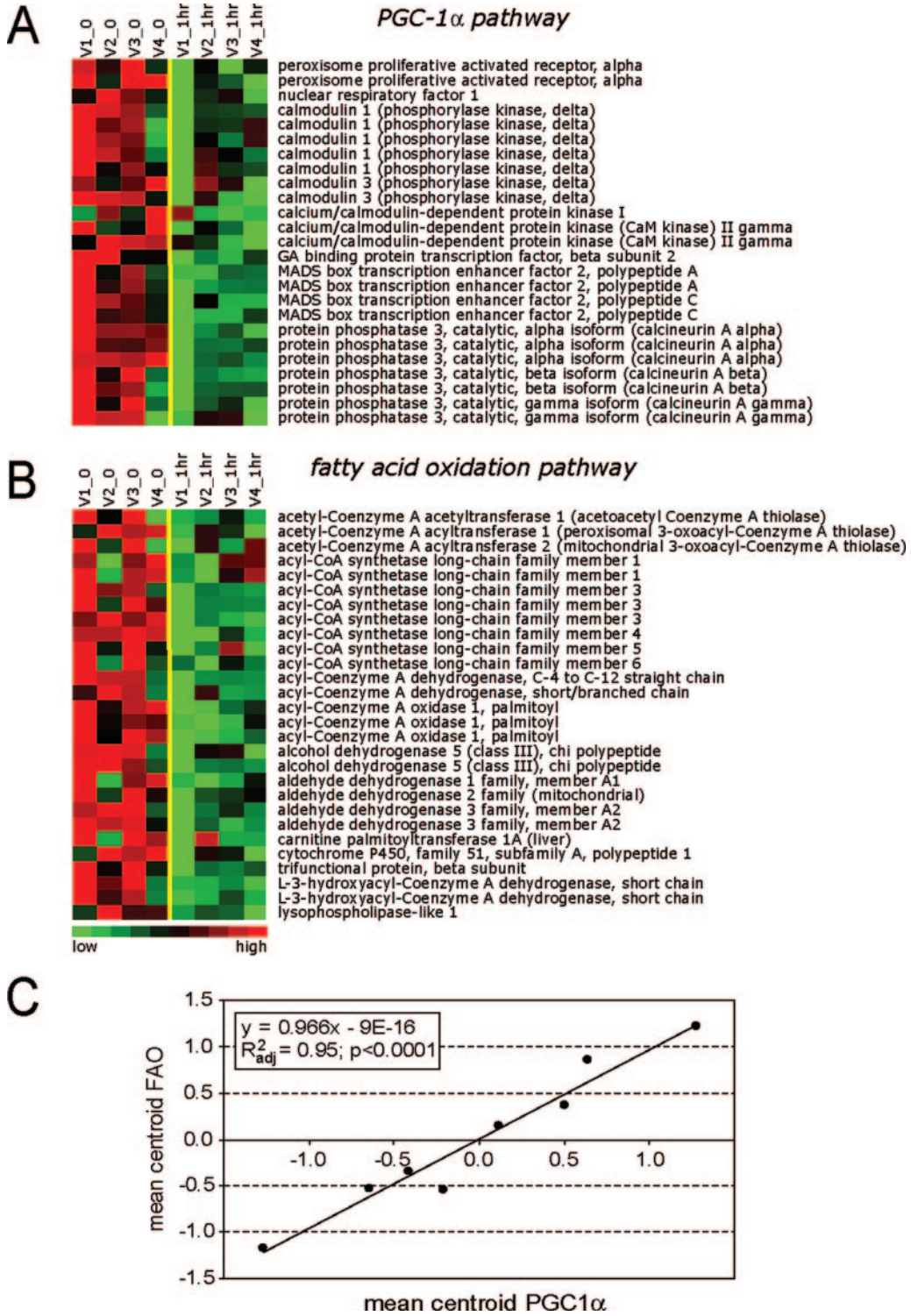

Figure 5. Results from Gene Set Enrichment Analysis (GSEA). Panel A: Down-regulation of peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α) pathway activity. Panel B: Down-regulation of fatty acid oxidation (FAO) pathway. Panel C: Strong correlation in expression of the PGC-1α genes and genes involved in FAO. The gene expression levels of the 25 significantly enriched PGC-1α probes and the 28 significantly enriched FAO probes were standardized to a mean of 0 and a variance of 1 across the 8 microarrays. The mean centroid vector is the mean of these 25 and 28 expression vectors, respectively. Expression levels are depicted as color scale from red (high expression) to green (low expression). Numbers (V1–V4; $n = 4$) identify individual subjects.

and rolling, while β_2-integrin (CD11b) facilitates subsequent firm adhesion and transmigration (17). L-selectin (CD62L) is constitutively expressed on the surface of leukocytes. Inflammatory stimulation via G-protein coupled receptors, as mimicked in our study by the chemoattractant fMLP, rapidly activates L-selectin (CD62L) by phosphorylation and subsequent cleavage of its ectodomain, increasing the binding

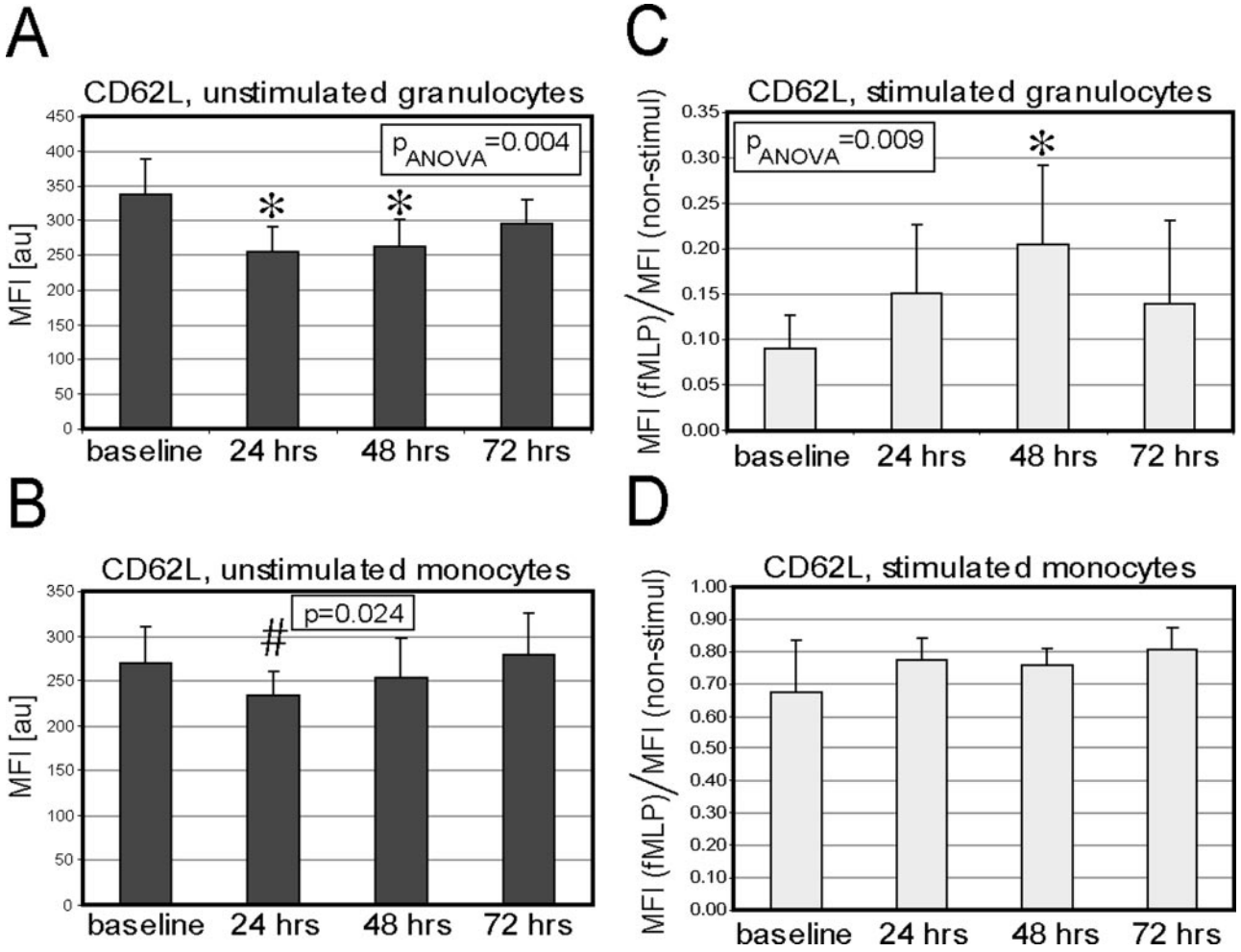

Figure 6. Expression of L-selectin (CD62L) in granulocytes (CD14+ cells) (Panel A; $P_{ANOVA} = 0.004$), and monocytes (CD15+ cells) (Panel B; #$P = 0.024$, paired *t*-test, comparison baseline versus 24h). Activation and shedding of granulocytes (Panel C; $P_{ANOVA} = 0.009$), and monocytes (Panel D; not significant) in response to *N*-formyl-methionyl-leucyl-phenylalanin (fMLP) stimulation. *$P < 0.05$ compared with baseline (Student-Newman-Keuls *post hoc* test). MIF = mean fluorescence intensity. Data represent mean ± SD ($n = 5$).

activity to the endothelium (17). Thus, our findings suggest that sevoflurane induces a resistance of leukocytes against noxious stimulation. Blockade of selectin-mediated leukocyte adhesion improves postischemic function in the heart (32), while genetically engineered mutations that prevent shedding of cell surface receptors markedly diminish the deleterious inflammatory response (33). In contrast to remote ischemic preconditioning of the limb, in our study small-dose sevoflurane did not decrease β_2-integrin (CD11b) expression or its shedding in response to the chemoattractant fMLP. This is in agreement with previous *in vitro* experiments showing that 0.5 MAC isoflurane inhibited shedding of L-selectin (CD62L), but not β_2-integrin (CD11b), in response to chemoattractants, whereas 1 MAC isoflurane affected both adhesion molecules (34). Although L-selectin (CD62L) was not among the differentially regulated transcripts in our human experiments, calmodulin, a calcium regulatory protein, which co-precipitates with L-selectin (CD62L) and tightly regulates its surface expression and function (35), was markedly down-regulated after sevoflurane inhalation (Fig. 5). This notion raises the interesting possibility that delayed alterations in L-selectin (CD62L) expression and function might be indirectly caused by reduced calmodulin expression. It has been traditionally thought that the biological effects of fast-acting anesthetic gases with low blood-gas solubility dissipate rapidly after exhalation. In contrast to this belief, our study clearly demonstrates that these gases continue to exert their potentially protective actions long after their physical clearance from the human body, consistent with published results in patients undergoing coronary artery bypass grafting surgery (36).

Gene Regulatory Control of Energy Metabolism in the Blood Mirrors Conditions in the Heart

We have recently shown that IV and volatile anesthetics differentially regulate the transcriptional response to cardiac surgery in human hearts (20). In particular, sevoflurane shifted the metabolic fuel preference away from fatty acid oxidation, which closely

correlated with improved postoperative cardiac function. Myocardial substrate metabolism critically affects cardiac function (37), and switching metabolic fuel preference away from fatty acid oxidation improves recovery after ischemia and even affects long-term outcome (38). The present study confirms similar metabolic changes in the blood after subanesthetic sevoflurane inhalation, and further suggests that the transcriptional changes in fatty acid oxidation may be due to down-regulation of the PGC-1α pathway. This nuclear receptor coactivator critically controls cellular energy metabolism, and thus determines oxygen consumption (39). Moreover, inhibition of the PGC-1α pathway was recently shown to reduce uptake of oxidized low-density lipoprotein into macrophages and to prevent their retention in atherosclerotic vessel walls, thereby stabilizing vulnerable plaques (40). Hence, modulation of the PGC-1α pathway, as observed after sevoflurane administration, may be a novel antiischemic and plaque-stabilizing strategy in perioperative medicine.

Limitations

The sample size of this study is small. However, a highly standardized protocol was used in healthy volunteers to overcome confounding variables. In this study, we used ranitidine to protect the participants from the potential hazard of pulmonary aspiration, and some previous reports suggest a role of histamine receptors in modifying expression of adhesion molecules (41,42). However, there was no transcriptional regulation in our control experiments after inhalation of 50% oxygen and administration of ranitidine alone. Also, changes in L-selectin expression exhibited the maximum at 24–48 h after sevoflurane inhalation. At this time, a single dose of ranitidine is cleared from the body, making a relevant effect on adhesion molecules unlikely. Moreover, the H_2 receptor antagonist, ranitidine, did not affect histamine-induced expression of adherence molecules (42), nor did it prevent histamine-induced leukocyte rolling (41). Future studies should help to precisely delineate the underlying signaling components. Whether the detected molecular evidence of late preconditioning by sevoflurane successfully translates into clinical benefits requires additional randomized controlled trials.

CONCLUSIONS

This study provides molecular evidence of late preconditioning after sevoflurane inhalation in healthy volunteers. Because the late phase lasts significantly longer than the early phase, its protective potential merits further investigation in the clinical arena.

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Gene Regulatory Control of Myocardial Energy Metabolism Predicts Postoperative Cardiac Function in Patients Undergoing Off-pump Coronary Artery Bypass Graft Surgery

Inhalational versus *Intravenous Anesthetics*

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Background: **Anesthetic gases modulate gene expression and provide organ protection. This study aimed at identifying myocardial transcriptional phenotypes to predict cardiovascular biomarkers and function in patients undergoing off-pump coronary artery bypass graft surgery.**

Methods: **In a prospective randomized trial, patients undergoing elective off-pump coronary artery bypass graft surgery were allocated to receive either the anesthetic gas sevoflurane (n = 10) or the intravenous anesthetic propofol (n = 10). Blood samples were collected perioperatively to determine cardiac troponin T, N-terminal pro–brain natriuretic peptide, and pregnancy-associated plasma protein A. Cardiac function was measured with transesophageal echocardiography and pulmonary artery thermodilution. Atrial biopsies were collected at the beginning and end of bypass surgery to determine gene expression profiles.**

Results: **N-terminal pro–brain natriuretic peptide and pregnancy-associated plasma protein A blood levels were decreased with sevoflurane treatment. Echocardiography showed preserved postoperative cardiac function in sevoflurane patients, which paralleled higher cardiac index measurements. N-terminal pro–brain natriuretic peptide release was predicted by sevoflurane-induced transcriptional reduction in fatty acid oxidation, whereas changes in cardiac index were predicted by preoperative gene activity of the peroxisome proliferator–activated receptor γ coactivator-1α pathway. Sevoflurane-mediated attenuation of transcripts involved in DNA-damage signaling and activation of the granulocyte colony-stimulating factor survival pathway predicted improved postoperative cardiac index and diastolic heart function, respectively.**

Conclusions: **Anesthetic-induced and constitutive gene regulatory control of myocardial substrate metabolism predicts postoperative cardiac function in patients undergoing off-pump coronary artery bypass graft surgery. The authors' analysis further points to novel cardiac survival pathways as potential therapeutic targets in perioperative cardioprotection.**

This article is accompanied by an Editorial View. Please see: Collard CD: "A razor may be sharper than an ax, but it cannot cut wood." ANESTHESIOLOGY 2007; 106:420–2.

Additional material related to this article can be found on the ANESTHESIOLOGY Web site. Go to http://www.anesthesiology.org, click on Enhancements Index, and then scroll down to find the appropriate article and link. Supplementary material can also be accessed on the Web by clicking on the "ArticlePlus" link either in the Table of Contents or at the top of the Abstract or HTML version of the article.

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OFF-PUMP coronary artery bypass graft (CABG) surgery emerged in recent years as a means to avoid the sequelae of extracorporeal circulation such as whole-body inflammatory response, coagulation disorders, and multiple organ dysfunction. However, this less invasive surgical technique still involves manipulation of the heart and coronaries and apparently does not eliminate irreversible myocardial injury.[1] The release of some cytokines is observed in off-pump CABG surgery to a similar degree as with cardiopulmonary bypass, although with delayed kinetics.[2] Therefore, improving the perioperative management by decreasing myocardial ischemia–reperfusion damage remains of paramount importance and would affect outcome.

Experimental work and recent data from clinical trials provide strong evidence that modern ether-derived halogenated anesthetic gases elicit pronounced protection against myocardial ischemia.[3] In contrast to intravenous anesthetics such as propofol, anesthetic gases exert a multitude of protective effects including pharmacologic preconditioning and postconditioning, two of the most powerful treatments to reduce infarct size and improving postischemic recovery.[4] In a double-blinded placebo-controlled study, brief administration of sevoflurane on

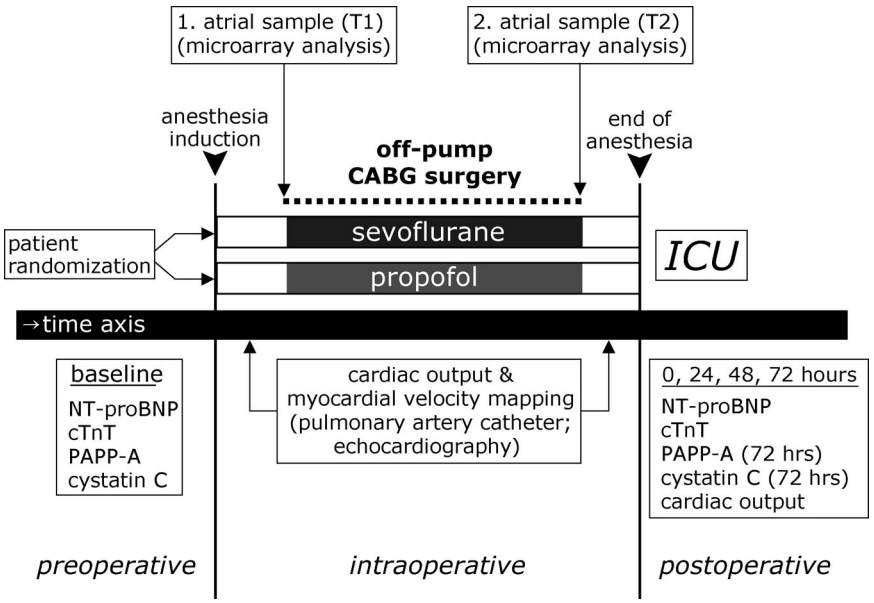

Fig. 1. Study protocol. CABG = coronary artery bypass grafting; cTnT = cardiac troponin T; ICU = intensive care unit; NT-proBNP = N-terminal pro brain natriuretic peptide; PAPP-A = pregnancy-associated plasma protein A.

the cardiopulmonary bypass immediately before induction of cardioplegia improved postoperative cardiac function[5] as well as long-term cardiovascular outcome in patients undergoing CABG surgery.[6] Moreover, administration of anesthetic gases throughout CABG surgery decreased perioperative troponin I release and the duration of intensive care unit and hospital stay.[7]

Using a genome-wide approach, we previously reported that distinct genetic programs are activated by anesthetic gases in isolated rat hearts, which attenuate the adverse consequences of prolonged ischemia.[4,8] This notion is consistent with the concept that anesthetic gases elicit a "second window of protection,"[9,10] which directly depends on altered gene expression.[3] Preliminary results from atrial biopsies suggest that similar genomic reprogramming may occur in patients.[6] To date, no direct comparisons between intravenous anesthetics and anesthetic gases have been performed in human hearts at the gene expression level. In accordance with the reported differences in cardioprotection between various anesthetics, we hypothesized that sevoflurane and propofol would elicit differential genomic responses to cardiac surgery in human hearts. Transcriptional changes were correlated to clinically important cardiovascular biomarkers and to physiologic parameters of cardiac function.

Materials and Methods

The institutional ethics committee of the University Hospital Zurich (Zurich, Switzerland) and the Triemli Hospital (Zurich, Switzerland) approved the study, and written informed consent was obtained from all patients. Twenty patients scheduled to undergo elective off-pump CABG surgery were enrolled between January and June 2005.

Study Criteria

Inclusion criteria were being scheduled for elective off-pump CABG surgery, three-vessel coronary artery disease, male sex, and age of 50–80 yr. Exclusion criteria were diabetes mellitus, concomitant noncardiac surgery, myocardial infarction less than 2 months before CABG surgery, elevated plasma concentrations for cardiac enzymes within 24 h before surgery, unstable angina, left ventricular ejection fraction less than 40%, hemodynamic instability with the need for medical or mechanical inotropic support, serum creatinine greater than 150 μM, and administration of corticosteroids or preconditioning-mimicking/blocking agents such as diazoxide, nicorandil, theophylline, and sulfonylurea drugs, respectively.

Study Protocol

The patients were randomly allocated to the sevoflurane (Sevorane; Abbott, Baar, Switzerland) or the propofol (Diprivan 2%; AstraZeneca, Zug, Switzerland) group (fig. 1). Fentanyl, midazolam, and rocuronium or pancuronium were administered before intubation. Anesthesia was maintained with midazolam, fentanyl, and remifentanil in all patients until the first atrial biopsy was taken (baseline biopsy at T1). Subsequently, anesthesia was maintained with the anesthetic gas sevoflurane or the intravenous anesthetic propofol until the second atrial biopsy was collected shortly before chest closing (biopsy at T2). Sevoflurane and propofol were adjusted to maintain blood pressure and heart rate within 20% of baseline values. During surgical manipulations on the heart, conversion to cardiopulmonary bypass was considered if one of the following criteria was present for at least 15 min: (1) cardiac index less than 1.5 $l \cdot min^{-1} \cdot m^{-2}$, (2) mixed central venous saturation less than 60%, (3) mean arterial pressure less than 50 mmHg, (4) ST-

segment alterations indicative of new myocardial ischemia, or (5) severe arrhythmias. After completion of bypass grafting, remifentanil infusion and repetitive doses of midazolam were used in both groups for sedation until extubation in the intensive care unit. Standard transesophageal echocardiography, tissue Doppler imaging, and hemodynamic measurements using pulmonary artery thermodilution were obtained (fig. 1). Patients from both groups received the same postoperative care.

Cardiac Surgery and Assessment of Myocardial Function

Median sternotomy and pericardiotomy were performed, and the first sample of the right atrium was immediately collected before preparing the grafts. During the surgical manipulation of the heart, hemodynamic stability was achieved by repeated doses of norepinephrine, nitroglycerin, positioning of the operating table, and sequential epicardial stimulation of the heart at a rate of 90 beats/min. After securing the operating field with the aid of a stabilizer (Octopus; Medtronic, Minneapolis, MN), the coronary artery was opened and an intracoronary shunt, which was withdrawn at the end of suturing, was introduced to preserve distal perfusion. The left mammary artery was used to revascularize the left anterior coronary artery. Venous grafts were used for the inferior myocardial wall, and the right internal mammary artery was used for the circumflex branches either through the transverse sinus behind the aorta and connected *in situ* to the circumflex branch, or, if too short, used as a T-graft from the left internal mammary artery. Proximal anastomoses were performed using the heart string device (Guidant, Indianapolis, IN). The quality of all bypasses was controlled by flow measurement (Medistim, Oslo, Norway).

Echocardiographic measurements were obtained before surgery and after completion of coronary bypass grafting. Standard transesophageal echocardiography and tissue Doppler imaging using a Philips SONOS 5500 system with an Omniplane III-TOE probe (Philips Medical Systems, Andover, MA) were performed by a single experienced examiner. The echocardiograms were stored digitally and analyzed *post hoc* without knowing the clinical data or group assignment. Left ventricular end-diastolic area and left ventricular end-systolic area were measured by manual planimetry of the area circumscribed by the leading edge of the endocardial border in the left ventricular transgastric midpapillary short axis view. Left ventricular end-diastolic area was determined as the largest left ventricular cross-sectional area after the R wave, and left ventricular systolic area was determined as the smallest left ventricular cross-sectional area after the T wave. Left ventricular fractional area change (percent) was calculated as $100 \times$ (left ventricular end-diastolic area $-$ left ventricular systolic area)/left ventricular end-diastolic area. Pulsed wave Doppler measurements of transmitral blood flow were obtained with the probe in the midesophageal four-chamber view after placing the sample volume between the tips of the mitral leaflets. Peak early mitral inflow velocity (E wave), deceleration time, peak atrial filling velocity (A wave), and E/A ratio were also measured. Assessment of color M-mode flow propagation was performed with the M-mode cursor aligned parallel to the left ventricular inflow. Main aliased velocity was determined after adjustment to obtain the longest flow column. Tissue Doppler imaging was also obtained from the midesophageal four-chamber view to quantify regional myocardial contractility. Myocardial velocities of the left ventricular septum, the lateral wall, and the mitral annulus were measured. Early systolic, early diastolic, and late diastolic peak velocities were determined using QLAB advanced quantification software version 2.0 (Philips Ultrasound, Bothell, WA). All echocardiographic measurements were recorded as the mean of three consecutive cardiac cycles. Concomitant hemodynamic measurements were obtained before surgery, after completion of coronary bypass grafting, 4 h after arrival in the intensive care unit, and at discharge from the intensive care unit using pulmonary artery thermodilution as the mean of three consecutive ice-water bolus injections.

Diagnosis of Adverse Events

Medical charts were reviewed, and the caregivers were interviewed daily for the occurrence of adverse events. Adverse events (as opposed to myocardial and renal injury markers determined at the end of the study) were diagnosed by the independently managing clinicians. Twelve-lead electrocardiograms were obtained postoperatively every day. The diagnosis of a new postoperative myocardial infarct required a new Q wave, persistent ST-T-segment changes as defined by Minnesota Codes,[11] and/or cardiac troponin T (cTnT) greater than 2.0 μg/l. The diagnosis of a cerebrovascular injury required the presence of clinical symptoms and/or positive computer-assisted tomography scan. The diagnosis of significant postoperative renal dysfunction required newly established postoperative hemodialysis or hemofiltration.

Determination of Cardiovascular Biomarkers

Blood samples were obtained preoperatively, at arrival in the intensive care unit, and 24, 48, and 72 h after surgery and were used to determine cTnT and N-terminal pro brain natriuretic peptide (NT-proBNP) plasma levels. Pregnancy-associated plasma protein A (PAPP-A) and cystatin C plasma levels were only determined preoperatively and after 72 h after surgery. The collected blood samples were stored at -80°C until analysis. The following parameters were determined using the Roche Modular Analytics P or the Roche Modular Analytics

E170 (Roche Diagnostics, Mannheim, Germany): NT-proBNP (electrochemiluminescence sandwich immunoassay)—sensitivity greater than 5 ng/l, intraassay and interassay coefficients of variance less than 3%, normal value (97.5th percentile for men aged > 50 yr) less than 334 ng/l; cTnT (electrochemiluminescence sandwich immunoassay)—sensitivity greater than 0.01 μg/l, intraassay and interassay coefficients of variance greater than 2.5%, normal value less than 0.01 μg/l. PAPP-A assays were purchased from Brahms, Henningsdorf, Germany (immunometric assay based on time resolved amplification cryptate emission)—sensitivity 0.004 U/l, intraassay and interassay coefficients of variance less than 2%, normal range (healthy male) less than 0.01 U/l. Cystatin C assays were purchased from Dako A/S, Goldstrup, Denmark (particle-enhanced turbidimetric assay)—sensitivity greater than 0.2 mg/dl, intraassay and interassay coefficients of variance less than 3.5%, normal range 0.63–1.61 mg/l.

Genomic Analysis of Human Atrial Samples

Microarray analysis was performed following the "minimum information about a microarray experiment" (MIAME) guidelines.[12] Total RNA was prepared from the frozen cardiac tissue using RNeasy Mini Kit (Qiagen AG, Basel, Switzerland). The quality of the isolated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE; 260 nm/280 nm ratio between 1.8 and 2.1 and 28S/18S ratio within 1.5–2). Total RNA samples (100 ng) were reverse-transcribed into double-stranded complementary DNA (cDNA) with a Two-Cycle cDNA Synthesis Kit (Affymetrix Inc., Santa Clara, CA). The double-stranded cDNA was purified using a Sample Cleanup Module (Affymetrix Inc.). The purified double-stranded cDNA were *in vitro* transcribed in the presence of biotin-labeled nucleotides using an IVT Labeling Kit (Affymetrix Inc.). The biotinylated complementary RNA (cRNA) was purified using a Sample Cleanup Module (Affymetrix Inc.), and its quality and quantity was determined using NanoDrop ND 1000 and Bioanalyzer 2100. Biotin-labeled cRNA samples (15 μg) were fragmented randomly to 35–200 bp at 94°C in Fragmentation Buffer (Affymetrix Inc.) and were mixed in 300 μl hybridization buffer containing a hybridization Control cRNA and Control Oligo B2 (Affymetrix Inc.), 0.1 mg/ml herring sperm DNA, and 0.5 mg/ml acetylated bovine serum albumin in 2-(4-morpholino)-ethane sulfonic acid (MES) buffer at pH 6.7. Each sample (for a total of 40 samples) was hybridized to an individual Affymetrix GeneChip Human Genome U_133 Plus 2.0 arrays for 16 h at 45°C (Affymetrix Inc.). Arrays were washed using an Affymetrix Fluidics Station 450 EukGE-WS2v5_450 protocol and scanned using an Affymetrix GeneChip Scanner 3000 (Affymetrix Inc.) at a resolution of 3 μm. Raw data processing was performed using the Affymetrix GCOS 1.2 software (Affymetrix Inc.), and all arrays were subjected to quality control according to the parameters established by Affymetrix, such as the assessment of average background and noise value, exogenously added prokaryotic hybridization controls, percent present calls, scaling factors, and internal control genes. Normalization and computation of expression values were performed using the robust multichip average method.[13] The microarray data are available at the Gene Expression Omnibus Database## under the series number GSE4386. For each patient, two atrial samples were collected and used for hybridization. No samples were pooled, *i.e.*, 40 samples corresponding to 40 chips were used in the final analysis. The global gene expression matrix containing the expression values of the 54,675 probe sets for all 40 cardiac samples was used as input for Gene Set Enrichment Analysis (GSEA).[14] GSEA considers predefined gene sets representing pathways of interest and determines whether the members of these sets are overrepresented in a list of genes that has been ordered by their correlations with a specific phenotype or class distinction. The output is a normalized enrichment score that represents a measure of the degree of enrichment of the gene set at the top (highly correlated) or bottom (highly inversely correlated) of the ordered gene list. The normalized enrichment score is used to produce a *P* value that measures the significance of the score itself. The gene sets used for the current analysis (a total of 547) were obtained from the GSEA home page*** or were manually curated. Additional information regarding this is available on the ANESTHESIOLOGY Web site at http://www.anesthesiology.org.

In a first analysis, the "GSEA phenotypes" were defined as follows: SEVO (t = T1), samples collected in the sevoflurane group at time T1; SEVO (t = T2), samples collected in the sevoflurane group at time T2; PROP (t = T1) and PROP (t = T2), samples collected in the propofol group at times T1 and T2, respectively. To define a standardized measure of gene expression (the mean centroid), we normalized the gene expression levels of the enriched genes within a pathway to a mean of 0 and a variance of 1 across all 40 samples. The mean centroid vector of a given pathway is computed as the mean of the normalized expression vectors of each gene in the pathway. A second GSEA analysis was performed using the computed fold changes from T1 to T2 for all Affymetrix probe sets. In this case, two phenotypes (SEVO and PROP) were defined.

GEO database. Available at: http://www.ncbi.nlm.nih.gov/geo/. Accessed March 8, 2006.

*** Available at: http:// www.broad.mit.edu/GSEA. Accessed October 30, 2005.

Validation of Chip Data Using Real-time Reverse-transcriptase Polymerase Chain Reaction

As an independent method of measuring levels of gene expression, real-time reverse-transcriptase polymerase chain reaction was performed for eight selected genes to confirm microarray data (table S1 on the ANESTHESIOLOGY Web site). For this purpose, randomly chosen transcripts with a wide range of fold changes (0.12–8.0), as obtained by gene chip analysis, were selected. First-strand cDNA was synthesized from 1 μg total RNA using Superscript II reverse transcriptase (Invitrogen, Basel, Switzerland) and oligo-dT as primer. Real-time reverse-transcriptase polymerase chain reaction quantification of the selected genes was performed on a Stratagene MX3000 real-time sequence detector instrument (Stratagene Europe, Amsterdam, The Netherlands) using the Brilliant SYBR green QPCR Master Mix (Stratagene Europe). Amplification reactions were conducted with an initial step at 90°C for 3 min followed by 20–35 cycles. Samples were run in triplicates, and ribosomal 18S (a constitutively expressed gene) was used as reference control. Predicted size of amplified products was confirmed by agarose gel electrophoresis.

Statistical Analysis

Sample size was calculated based on the results for NT-proBNP reported previously.[5] For biochemical and physiologic parameters, two-way analysis of variance for repeated measures followed by appropriate multiple comparison procedures (Bonferroni *t* test) was used to evaluate differences over time between groups. An unpaired *t* test was used to compare groups at identical time points, and a paired *t* test was used to compare within groups over time. All other data were analyzed using unpaired *t* tests for parametric data or Mann–Whitney tests for nonparametric data. Categorical variables were summarized by proportions and compared using the Fisher exact test or the chi-square test, if appropriate. *P* values were multiplied by the number of comparisons that were made (Bonferroni correction), and corrected $P < 0.05$ was considered significant. Data are given as mean ± SD or median and quartiles, if appropriate. Forward stepwise linear regression (with F-to-Enter = 4.000 and F-to-Remove = 3.996) was applied using functional/biochemical outcome variables as dependent variables and gene expression measures (mean centroids or fold changes) and clinical data as the independent variables. Adjusted squared correlation coefficients (R^2_{adj}) were reported. Analyses were performed using SigmaStat Version 2 (SPSS Science, Chicago, IL) and StatView version 5 (SAS Institute, Chicago, IL).

Results

Demographics, Perioperative Data, and Clinical Outcome

Patient characteristics and perioperative data are listed in table 1. The sevoflurane and propofol groups were similar with regard to all preoperative data including medical therapy and comorbidity. Intraoperative data were comparable between groups except for the longer anesthesia and surgery time and the smaller amount of administered remifentanil in the sevoflurane group. The cumulative doses of norepinephrine and nitroglycerin used to stabilize hemodynamics during the surgical manipulations were similar in both groups (table 1). None of the patients required conversion to cardiopulmonary bypass or had postoperative myocardial infarction, cerebrovascular injury, or renal damage. No postoperative mechanical or medical inotropic support was needed.

Biomarkers for Contractile Dysfunction (NT-proBNP) and Coronary Plaque Instability (PAPP-A) Indicate Superior Protection by Sevoflurane

Sevoflurane markedly reduced postoperative NT-proBNP release (fig. 2A). Mean peak postoperative NT-proBNP was 3,442 ± 1,458 ng/l in propofol patients compared with 1,392 ± 881 ng/l in sevoflurane patients ($P = 0.0013$). In contrast, no difference was observed in postoperative cTnT release (fig. 2B). However, two propofol patients but none of the sevoflurane patients had a peak postoperative cTnT concentration greater than 0.65 μg/l, indicative of major perioperative myocardial damage. None of the patients experienced postoperative deterioration of renal function (postoperative cystatin C plasma concentrations in sevoflurane patients: 0.63 ± 0.16 mg/l *vs.* propofol patients: 0.64 ± 0.18 mg/l; $P = 0.55$; fig. 2C). Preoperative baseline PAPP-A plasma concentrations were increased in all patients indicating an active inflammatory process in atherosclerotic plaques. Sevoflurane but not propofol abolished the postoperative PAPP-A elevation (mean change in PAPP-A: −1.10 ± 1.10 mU/l in sevoflurane patients *vs.* +6 ± 1.05 mU/l in propofol patients; $P < 0.001$; fig. 2D).

Transesophageal Echocardiography and Hemodynamic Measurements Show Improved Postoperative Myocardial Function after Sevoflurane Anesthesia

Sevoflurane but not propofol preserved inotropy (predominantly lateral wall) and early diastolic lusitropic function (annulus and septum) after CABG surgery (table 2) and thereby successfully maintained global early postoperative cardiac function as determined by cardiac index measurements (table 3). Sevoflurane patients, as opposed to propofol patients, also exhibited increased postoperative ejection fractions compared with preop-

Table 1. Patient Characteristics and Perioperative Data

	Propofol Group (n = 10)	Sevoflurane Group (n = 10)
Age, yr	66.9 ± 7.3	65.2 ± 7.6
Euroscore	2.9 ± 1.5	3.0 ± 2.4
Parsonnet score	4.9 ± 5.0	4.0 ± 4.4
Preoperative ejection fraction, %	66 ± 10 (45–75)	60 ± 13 (42–76)
Previous myocardial infarction, No. of patients	2	2
Preoperative medication, No. of patients		
β Blocker	10	9
Ca^{2+} blocker	3	5
Nitrates	4	5
ACEI	2	3
ATA	4	4
Statins	6	5
Diuretics	6	7
No. of grafts	3.4 ± 0.8 (2–5)	3.9 ± 0.7 (3–5)
Anesthetic drugs		
Propofol, mg*	1,128 ± 364	—
Sevoflurane, vol% end-tidal	—	1.9 ± 0.5 (0.6–2.5)
Midazolam, mg	22.4 ± 6.3	24.5 ± 6.1
Fentanyl, mg	1.4 ± 0.4	1.2 ± 0.4
Remifentanil, mg	4.4 ± 1.5	2.6 ± 0.7‡
Vasoactive drugs		
Norepinephrine, μg	661 ± 936 (0–3,400)	662 ± 881 (0–2,510)
Nitroglycerin, mg	4.84 ± 3.41 (0.6–10)	5.45 ± 2.63 (0.7–8.6)
Volume replacement, ml†	4,272 ± 1,887 (1,830–6,677)	5,090 ± 1,540 (3,430–8,400)
Surgery time, min	222 ± 37	266 ± 44‡
Anesthesia time, min	295 ± 46	352 ± 52‡
Elapsed time between first and second atrial biopsy, min	203 ± 39	174 ± 37

Data are mean ± SD (range), if not otherwise indicated.

* Propofol infusion rate during maintenance of anesthesia: 0.5–2.5 mg · kg^{-1} · min^{-1}. † Total intraoperatively replaced volume (blood products, colloids, and crystalloids). ‡ $P < 0.05$ compared with propofol.

ACEI = angiotensin-converting enzyme inhibitor; ATA = angiotensin II antagonist.

erative values (table 2). Late postoperative hemodynamic measurements obtained in the intensive care unit showed an increase in postoperative cardiac index compared with preoperative measurements in both sevoflurane and propofol patients. However, this increase was more pronounced in sevoflurane patients and further accompanied with a reduced systemic vascular resistance (table 3).

Gene Expression Profiling Shows Pronounced Responses to Off-pump CABG Surgery and Unveils Subtle Differences in Background Energy Metabolism among Patients

For each patient, two atrial samples were collected, the first sample immediately after chest opening (110 ± 45 min after induction of anesthesia; T1) and the second sample shortly before chest closing (180 ± 40 min after obtaining

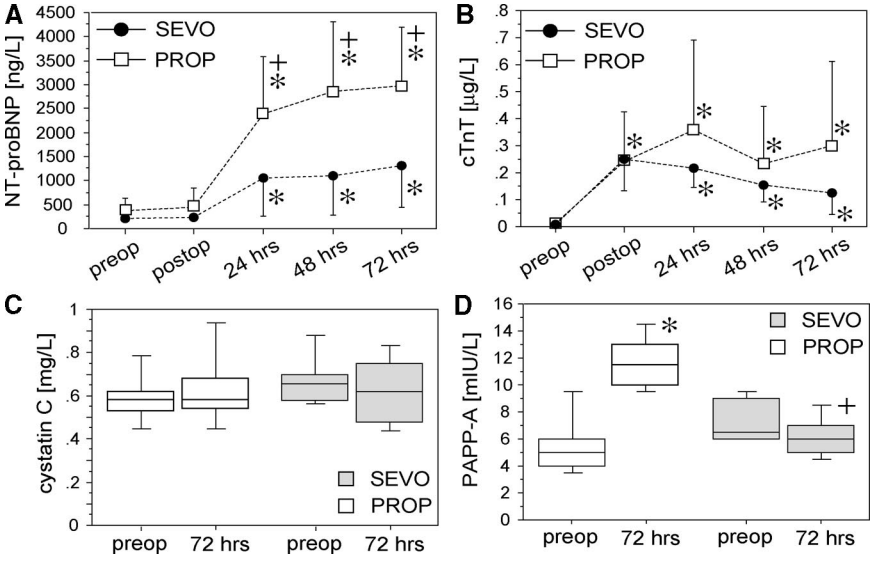

Fig. 2. Cardiovascular biomarkers. Perioperative levels of N-terminal pro brain natriuretic peptide (NT-proBNP) were significantly (time effect $P < 0.001$; treatment effect $P = 0.002$) more elevated in the propofol (PROP) group as compared with the sevoflurane (SEVO) group (*A*). No difference between groups was found in cardiac troponin T (cTnT) (*B*) and in cystatin C (*C*), whereas pregnancy-associated plasma protein A (PAPP-A) was exclusively increased (time effect $P < 0.001$; treatment effect $P = 0.02$) postoperatively in the PROP group (*D*). * Significantly increased compared with baseline values ($P < 0.05$; multiple comparison procedure, Bonferroni-corrected *t* test). + Significantly different between groups ($P < 0.05$; multiple comparison procedure, Bonferroni-corrected *t* test). Data are mean ± SD in *A* and *B*, and medians and 25th and 75th percentile in *C* and *D*.

Table 2. Echocardiographic Parameters

	Before CABG		After CABG	
	Propofol	Sevoflurane	Propofol	Sevoflurane
LV-ESA, cm^2	9.2 ± 3.2	9.6 ± 2.3	7.9 ± 3.4	8.2 ± 3.2
LV-EDA, cm^2	18.3 ± 3.4	18.4 ± 3.2	16.9 ± 4.8	18.8 ± 6.0
LV-FAC, %	50 ± 10	50 ± 10	51 ± 9	56 ± 10*
Diastolic inflow				
E wave, cm/s	62.3 ± 15.2	54.5 ± 16.2	52.1 ± 24.3	50.9 ± 15.2
A wave, cm/s	46.7 ± 20.3	45.3 ± 23.4	40.9 ± 19.1	45.1 ± 15.6
E/A ratio	1.6 ± 0.9	1.5 ± 0.9	1.7 ± 1.4	1.3 ± 0.5
Peak mitral annulus velocity				
Systolic, cm/s	3.8 ± 1.3	3.2 ± 1.0	2.9 ± 1.1	4.2 ± 1.5*
Early diastolic, cm/s	3.7 ± 2.3	3.6 ± 2.2	2.1 ± 1.4*	3.9 ± 1.6†
Late diastolic, cm/s	4.2 ± 2.4	3.5 ± 1.5	3.8 ± 1.4	4.4 ± 1.4*
Myocardial velocity septal				
Systolic, cm/s	2.1 ± 0.8	2.0 ± 1.2	2.1 ± 0.9	3.1 ± 1.8*
Early diastolic, cm/s	2.7 ± 1.0	2.3 ± 0.8	1.7 ± 0.5*	2.9 ± 1.3†
Late diastolic, cm/s	2.4 ± 1.1	2.0 ± 1.1	2.5 ± 1.2	2.3 ± 1.4
Myocardial velocity lateral				
Systolic, cm/s	2.4 ± 1.1	2.3 ± 1.2	1.5 ± 0.5	2.5 ± 0.8†
Early diastolic, cm/s	2.3 ± 1.7	2.4 ± 0.6	1.2 ± 0.6*	2.6 ± 0.9†
Late diastolic, cm/s	2.2 ± 1.2	2.1 ± 1.0	1.5 ± 0.7	1.8 ± 1.0

Data are mean ± SD.

* $P < 0.05$ with values before coronary artery bypass grafting (CABG). † $P < 0.05$ compared with propofol.

A wave = peak late mitral inflow velocity; E wave = peak early mitral inflow velocity; EDA = end-diastolic area; ESA = end-systolic area; FAC = fractional area change; LV = left ventricular.

the first sample; T2). Sample T1 reflects the background gene expression profile before exposure to sevoflurane or propofol (constitutive background activity), whereas sample T2 mirrors the gene responses to surgery in the two anesthetic groups. The existence and direction of gene expression changes were reliably detected by the microarray technology, as confirmed by real-time quantitative polymerase chain reaction (tables S1 and S2 on the ANESTHESIOLOGY Web site). As a first step, the single genes, which were differentially regulated over time (T1–T2) between the two anesthetic groups, were determined (see fig. S1, showing the 50 top-ranked up- and down-regulated genes, on the ANESTHESIOLOGY Web site). Off-pump CABG surgery induced pronounced changes in genes involved in transcriptional regulation and proinflammatory and antiinflammatory actions. Next, the comparison of the differentially and jointly regulated pathways over time (T1–T2) in sevoflurane- and propofol-treated patients showed a similar number of enriched pathways in propofol and sevoflurane patients (fig. 3A and table 4). At T1, GSEA further detected subtle differences (approximately 15%) in activity of genes involved in the peroxisome proliferator–activated receptor γ coactivator 1α (PGC-1α) pathway among patients (normalized enrichment score = 1.37; nominal P value = 0.04, false discovery rate q value = 0.26; fig. 3B). As expected, the PGC-1α pathway correlated with genes involved in oxidative phosphorylation ($R^2_{adj} = 0.71$, $P < 0.00001$) and fatty acid metabolism (uptake and oxidation) ($R^2_{adj} = 0.70$, $P <$

Table 3. Hemodynamic Data

	Before CABG		After CABG		ICU		ICU Discharge	
	Propofol	Sevoflurane	Propofol	Sevoflurane	Propofol	Sevoflurane	Propofol	Sevoflurane
HR, beats/min	57 ± 9	54 ± 6	89 ± 11*	84 ± 7*	91 ± 14*	86 ± 11*	86 ± 11*	82 ± 7*
MAP, mmHg	81 ± 11	82 ± 8	70 ± 24	73 ± 8	76 ± 7	74 ± 8	78 ± 6	77 ± 10
MPAP, mmHg	19 ± 7	24 ± 20	20 ± 7	22 ± 4	22 ± 5	22 ± 6	18 ± 4	17 ± 4
CVP, mmHg	8 ± 6	8 ± 5	8 ± 5	11 ± 4	6 ± 2	9 ± 3	5 ± 2	7 ± 4
PCWP, mmHg	10 ± 5	11 ± 3	10 ± 5	11 ± 4	8 ± 4	9 ± 2	8 ± 4	9 ± 3
CI, $l \cdot min^{-1} \cdot m^{-2}$	3.4 ± 0.6	3.5 ± 0.9	2.9 ± 0.5*	3.8 ± 1.1†	3.9 ± 0.7‡	5.0 ± 1.0*†‡	3.7 ± 0.9‡	4.7 ± 0.7*†‡
SVR, $dyn \cdot s \cdot cm^{-5}$	919 ± 249	928 ± 296	985 ± 237	702 ± 171*†	745 ± 99	558 ± 94*†	846 ± 179	627 ± 174*†
PVR, $dyn \cdot s \cdot cm^{-5}$	116 ± 58	168 ± 222	150 ± 73	120 ± 39	145 ± 67	109 ± 38	134 ± 94	65 ± 39

Data are mean ± SD.

* $P < 0.05$ compared with values before coronary artery bypass grafting (CABG). † $P < 0.05$ compared with propofol. ‡ $P < 0.05$ compared with values after CABG.

CI = cardiac index; CVP = central venous pressure; HR = heart rate (hearts were paced at 90 beats/min during CABG and the early postoperative period); ICU = intensive care unit; MAP = mean arterial pressure; MPAP = mean pulmonary arterial pressure; PCWP = pulmonary capillary wedge pressure; PVR = pulmonary vascular resistance; SVR = systemic vascular resistance.

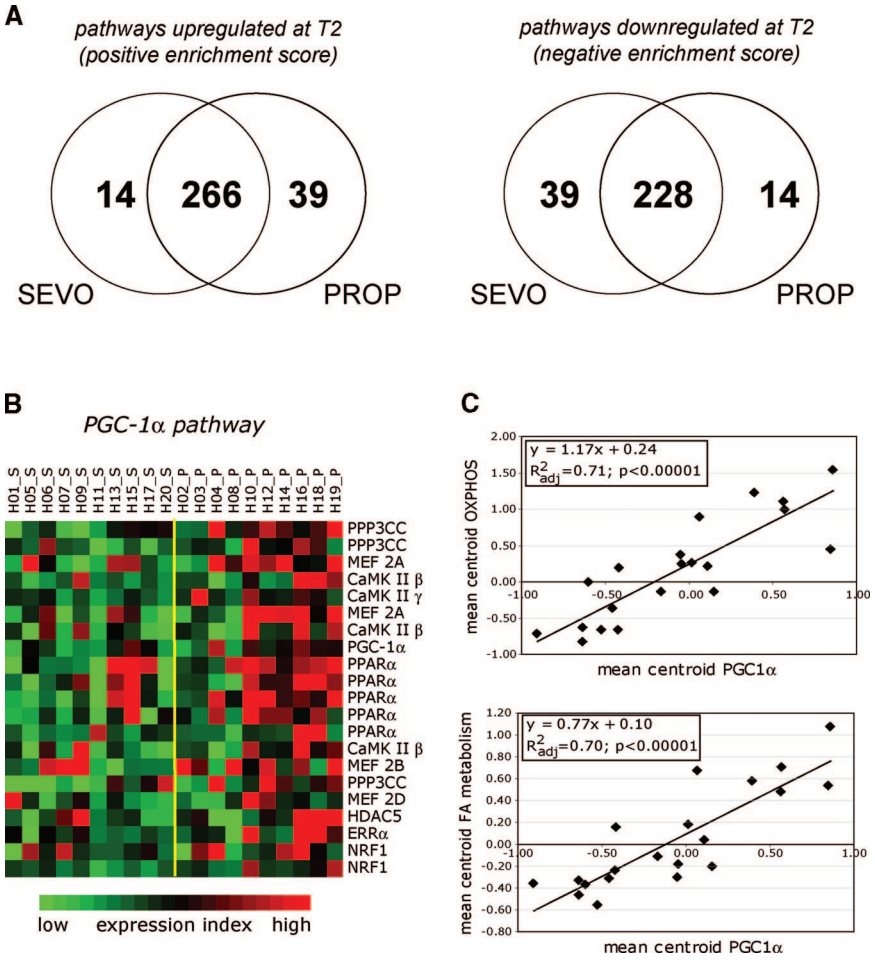

Fig. 3. (*A*) Pathways (gene sets) up-regulated and down-regulated at T2 in response to off-pump coronary artery bypass graft surgery in the two anesthetic treatments. The Venn diagrams show the number of enriched pathways (see also table 4). (*B*) Peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1α) pathway activity at baseline (t = T1). Each numbered square indicates the expression value of the indicated transcript in a specific patient. Subtle differences (approximately 15%) in PGC-1α activity were detected among patients. (*C*) PGC-1α pathway regulates genes involved in oxidative phosphorylation (OXPHOS) and fatty acid (FA) metabolism and was therefore used to express background energy metabolism in the heart (see also fig. S2 on the ANESTHESIOLOGY Web site). CaMK II β = calcium/calmodulin-dependent protein kinase II β; CaMK II γ = calcium/calmodulin-dependent protein kinase II γ; ERRα = estrogen-related receptor α; HDAC5 = histone deacetylase 5; MEF 2A = myocyte enhancer factor 2A; MEF 2B = myocyte enhancer factor 2B; MEF 2D = myocyte enhancer factor 2D; NRF1 = nuclear respiratory factor 1; PPARα = peroxisome proliferator activated receptor α; PPP3CC = calcineurin A γ; PROP = propofol patients; SEVO = sevoflurane patients.

0.00001) (fig. 3C) and hence was subsequently used in the multivariate analysis as an indicator of the myocardial background energy metabolism (fig. S2 on the ANESTHESIOLOGY Web site).

Sevoflurane Favorably Modifies the Transcriptional Responses to Off-pump CABG Surgery

In accordance with the study protocol, short-term changes in gene expression between T1 and T2 should be due to the different anesthetics used. To specifically determine the genomic effects of the anesthetics and to correct for the differences in baseline transcriptional activity, GSEA was applied to fold changes in gene expression between T1 and T2. From this analysis, three major pathways emerged, including fatty acid oxidation (normalized enrichment score = 1.53; nominal *P* value = 0.02, false discovery rate q value = 0.33), DNA-damage signaling (normalized enrichment score = 1.68; nominal *P* value = 0.02, false discovery rate q value = 0.11), and granulocyte-colony stimulating factor (G-CSF) survival pathway (normalized enrichment score = −1.79; nominal *P* value = 0.00, false discovery rate q value = 0.10). Compared with propofol, the anesthetic gas sevoflurane reduced transcriptional activity of genes involved in fatty acid oxidation (*t* test *P* = 0.008) and DNA-damage signaling (*t* test *P* = 0.002) but activated the G-CSF survival pathway (*t* test *P* = 0.017) (fig. 4; for complete list of transcripts, see table S3 on the ANESTHESIOLOGY Web site). Interestingly, the lower activity of transcripts involved in fatty acid oxidation, as observed in sevoflurane patients, was closely correlated with low DNA-damage signaling ($P < 0.001$) and high G-CSF survival pathway activity ($P = 0.006$), implying a possible link between the metabolic phenotype and cardioprotection (fig. 5).

Anesthetic-induced and Constitutive Gene Regulatory Control of Myocardial Energy Metabolism Predicts Postoperative Cardiac Function in Patients Undergoing Off-pump CABG Surgery

Next, we correlated the anesthetic-induced short-term transcriptional changes and the observed differences in constitutive background energy metabolism to postoperative cardiac function. Cardiac function was measured with three independent methods, including NT-proBNP, transesophageal echocardiography, and pulmonary artery thermodilution. Forward stepwise linear regression analysis was applied to determine the best predictors of postoperative cardiac function. Figure 6 shows the regression plots with the best predictors of NT-proBNP, cardiac index, and

Table 4. Induced (Positive NES) and Repressed (Negative NES) Pathways in Off-Pump CABG Surgery

		SEVO			PROP		
GSEA Pathway	Description	NES	Nom. *P* Value	FDR q Value	NES	Nom. *P* Value	FDR q Value
etsPathway (Biocarta)	The Ets transcription factors promote macrophage differentiation	1.69	0.00	0.14	1.94	0.00	0.00
stressPathway (Biocarta)	Tumor necrosis factor–related inflammation and stress-activated kinases	1.67	0.00	0.13	1.69	0.00	0.02
CR_transcription_factors[51]	Cancer related genes that are also transcription factors	1.61	0.00	0.25	1.83	0.00	0.02
MAPK_Cascade (Biocarta)	Mitogen-activated protein kinase pathway	1.56	0.03	0.18	1.67	0.00	0.02
SERUM_INDUCED_MKL_INDEP_SRF[52]	Serum inducible genes identified as a subset of serum response factor (SFR) target genes that are MKL (SRF-specific transcriptional coactivator) independent	1.56	0.00	0.19	1.81	0.00	0.02
LEM_HSC[53]	Stem cell molecular signature	1.54	0.02	0.19	1.78	0.00	0.01
IL6Pathway (Biocarta)	IL-6–related kinases and transcription factors	1.57	0.02	0.19	1.57	0.00	0.06
G-CSF (manually curated)	G-CSF survival pathway	1.46	0.02	0.24	1.52	0.02	0.07
CR_repair[51]	Cancer-related genes involved in DNA repair	−1.58	0.00	0.32	−1.62	0.00	0.20
IL7Pathway (Biocarta)	B- and T-cell development and proliferation	−1.35	0.03	0.68	−1.50	0.00	0.43
atrbrcaPathway (Biocarta)	Double-stranded break repair	−1.52	0.03	0.36	−1.58	0.02	0.29
Fatty_acid_metabolism (GenMAPP)	Fatty acid metabolism	−1.48	0.04	0.14	−1.24	0.1	0.48
OXPHOS (GenMAPP)	Respiratory chain enzymes	−1.41	0.03	0.20	−1.32	0.15	0.43

Fatty acid metabolism and oxidative phosphorylation (OXPHOS) were significantly enriched ($P < 0.05$) in the SEVO group only.

FDR = false discovery rate (FDR is the estimated probability that a set with a given NES represents a false positive finding); G-CSF = granulocyte colony-stimulating factor; GSEA = Gene Set Enrichment Analysis*; IL-6 = interleukin 6; MAPK = mitogen-activated protein kinase; MKL = myocardin-related family of proteins (myocardin, megakaryoblastic leukemia 1, and megakaryoblastic leukemia 2); NES = normalized enrichment score (positive NES indicates up-regulation at T2; negative NES indicates down-regulation at T2); nom. = nominal; PROP = propofol patients; SEVO = sevoflurane patients; SRF = serum response factor.

* For pathway information, refer to Web Enhancement and http://www.broad.mit.edu/GSEA (accessed October 30, 2005).

peak diastolic velocity, a marker of left ventricular relaxation. Interestingly, the G-CSF survival pathway consistently predicted left ventricular diastolic function at different anatomical sites in the hearts. No correlation was found between transcriptional phenotypes and peak systolic velocities. PAPP-A and cTnT did not correlate with cardiac function and transcriptional phenotypes. Together, these data indicate that short-term (by anesthetics) and long-term (constitutive) gene regulatory control of myocardial energy metabolism predicts postoperative cardiac recovery.

Discussion

Here we show for the first time on a genome-wide basis that anesthetics differentially modulate the transcriptional response to cardiac surgery in human hearts. The volatile ether sevoflurane, as compared with the intravenous anesthetic propofol, decreased fatty acid oxidation, which tightly associated with reduced DNA-damage signaling and enhanced activation of the G-CSF survival pathway. These favorable transcriptional changes closely correlated with and predicted improved cardiac functional recovery, as determined by NT-proBNP release, cardiac index, and peak diastolic velocity. However, beside these rather acute transcriptional effects, chronic preoperative background activity of PGC-1α pathway–regulated genes involved in oxidative phosphorylation and fatty acid metabolism, independently predicted postoperative cardiac function. Collectively, our data imply that gene regulatory control of myocardial energy metabolism, either as constitutive background activity or as short-term therapeutic target, plays a pivotal role in perioperative cardioprotection.

We used high-density oligonucleotide microarrays to assess global changes in cardiac gene expression that

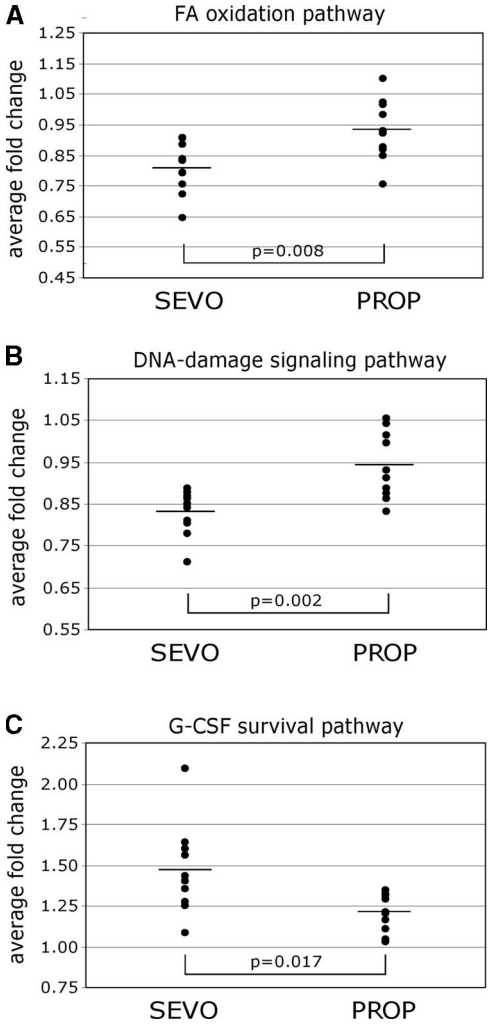

Fig. 4. Anesthetic-induced modulation of gene expression. Using fold changes in gene expression between T1 and T2, Gene Set Enrichment Analysis identified three major pathways, which were differentially regulated by sevoflurane (SEVO) and propofol (PROP). (*A*) Fatty acid (FA) oxidation pathway. (*B*) DNA-damage signaling pathway. (*C*) Granulocyte colony-stimulating factor (G-CSF) survival pathway. The average fold change of all enriched transcripts in the respective pathways was calculated (table S3 on the ANESTHESIOLOGY Web site). The individual *dots* represent changes in pathway activity of each patient. *Horizontal lines* indicate mean values.

result from two widely used anesthetics to gain insight into the underlying mechanisms of perioperative cardioprotection. Based on previously reported differences between the cardioprotective effects of anesthetic gases and intravenous anesthetics,[5,7,15–17] we sought for differential genomic responses in human hearts, which could be correlated to biochemical and functional outcome measures. To broaden the applicability of our results, off-pump CABG surgery was used as a model of human myocardial ischemia, which more closely mimics the situation of general surgical patients experiencing ischemia during noncardiac surgery. To assess the wide spectrum of interacting genes and the complex redundant and antagonistic patterns of transcriptional activation and suppression, we used GSEA, a sophisticated tool for pattern recognition.[14] Single gene approaches are limited to assessing complex pathophysiologic networks and miss important effects on entire pathways. In contrast, GSEA evaluates the microarray data on the level of functionally related genes based on previous biologic knowledge. The unique concept underlying GSEA relates to the fact that even small but coordinate changes in a majority of genes encoding members of a pathway (gene set) may dramatically alter the flux through the pathway. Using this more holistic approach, we were able to track the molecular footprints of anesthetics in the myocardium on a genome-wide basis and to gain novel insights into the mechanisms of perioperative cardioprotection.

In this study, we identified gene regulatory control of myocardial energy metabolism as the major determinant of postoperative cardiac function. A large body of literature supports the concept that myocardial substrate metabolism critically affects cardiac function in the acute and chronic disease state,[18] and that therapeutic strategies switching the metabolic fuel preference away from fatty acid oxidation can indeed improve contractile recovery after ischemia and affect long-term outcome.[19] Under normoxic conditions, the heart predominantly (approximately 60–80%) uses fatty acids as fuel source.[18] However, cardiac substrate utilization is markedly altered during ischemia with glucose as the preferred fuel. Conversely, in the postischemic myocardium, fatty acid oxidation prevails and nearly provides 100% of the cellular adenosine triphosphate (ATP) requirements.[20] Although we did not observe transcriptional changes in glucose transport, glycolysis, or glucose oxidation pathways, the reduction in fatty acid oxidation, *per se* leads to a relative increase in glucose utilization *via* activation of the pyruvate dehydrogenase complex (the glucose–fatty acid cycle by Randle[21]). Favoring the energetically economical glucose (3.17 ATP/oxygen molecule) over fatty acid oxidation (2.83 ATP/oxygen molecule) may be particularly beneficial in the diseased and mechanically stressed heart with only limited oxygen supply. Therefore, high-level fatty acid oxidation either by propofol anesthesia or as constitutive metabolic phenotype may put the heart at higher risk of postoperative contractile dysfunction.

Our study now demonstrates that this protective metabolic phenotype can be induced on a short-term basis in human hearts using anesthetic gases. We previously reported that isoflurane, another halogenated ether, profoundly reduced fatty acid oxidation in an *in vitro* rat heart model (fig. S3 on the ANESTHESIOLOGY Web site),[8] which is consistent with positron emission tomography results from isoflurane-anesthetized mice exhibiting strong and selective myocardial glucose uptake.[22] Likewise, our study patients with constitutive cardiac downregulation of the PGC-1α pathway may have shifted the

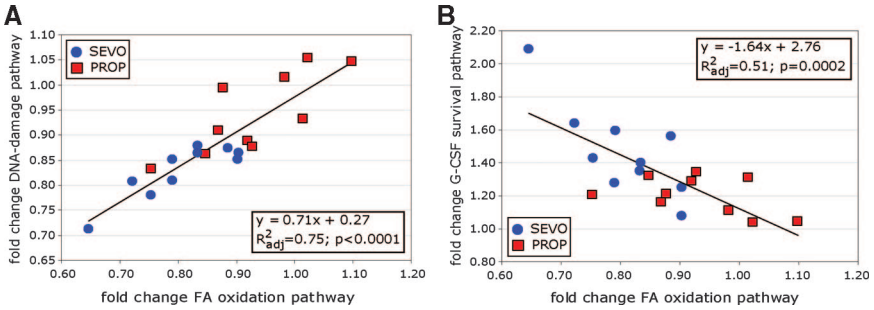

Fig. 5. Relation between anesthetic-induced metabolic changes in fatty acid (FA) oxidation and DNA-damage signaling (*A*), and granulocyte colony-stimulating factor (G-CSF) survival signaling (*B*), respectively. PROP = propofol patients; SEVO = sevoflurane patients.

metabolic fuel preference away from fatty acid oxidation and thus exhibited reduced mitochondrial respiration and oxidative phosphorylation gene activity. The PGC-1α pathway encloses the transcription factors peroxisome proliferator–activated receptor α, estrogen-related receptor α, the nuclear respiratory factors, and the transcriptional coactivator PGC-1α, which critically orchestrate control of cellular energy metabolism.[23] Although the PGC-1α pathway promotes a high energy state with increased contractility,[24] the PGC-1α–induced high ATP turnover may be detrimental under oxygen restriction.[25] Accordingly, hearts lacking peroxisome proliferator–activated receptor α, a key transcription factor of the PGC-1α pathway, exhibit improved ischemic tolerance, whereas hearts with peroxisome proliferator–activated receptor α overexpression only recover poorly after ischemia–reperfusion because of their inability to switch energy substrate.[26] Interestingly, reduced mitochondrial respiration resembles a state of metabolic hibernation, previously identified as a typical feature of the preconditioned state.[27,28] On the other side, anesthetic gases activate ATP-dependent potassium channels,[29,30] key players in the preconditioning process, which are tightly regulated by intermediary metabolites.[31] Together, our data shed new light on the intricate interplay among cellular metabolism, preconditioning, and perioperative cardioprotection and further suggest that modulation of the PGC-1α pathway may be used as a novel anti-ischemic strategy.

DNA-damage signaling and the G-CSF survival pathway closely correlated with fatty acid oxidation. An important question raised by our findings relates to the possible links between these pathways. In the case of DNA-damage signaling, the correlation may merely reflect the protection resulting from metabolic fuel shift. Among others, this pathway encloses the proinflammatory tumor necrosis factor α and the tumor suppressor p53 and the ataxia telangiectasia-mutated (a protein kinase that

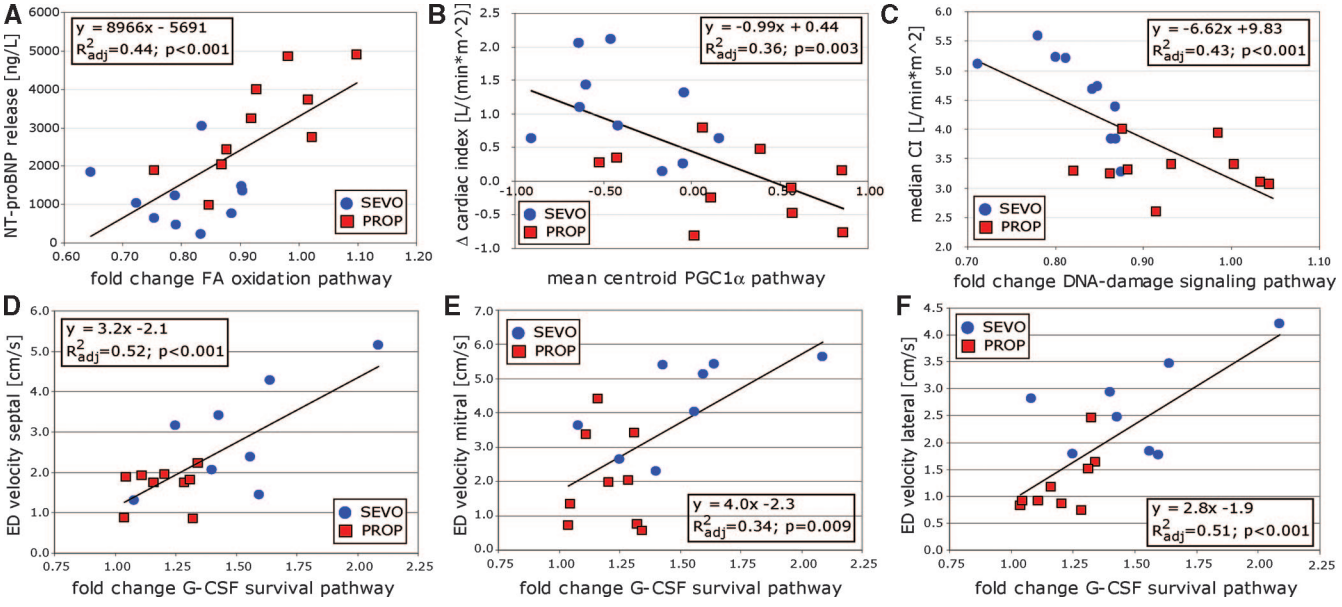

Fig. 6. Regression plots between transcriptional phenotypes and postoperative cardiac function. Multivariate analysis including the explanatory variables peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α) pathway (an index of long-term energy metabolism) and the anesthetic-induced short-term changes in gene expression was used to determine the best predictors of postoperative cardiac function. (*A*) Perioperative changes in N-terminal pro brain natriuretic peptide (NT-proBNP) were best predicted by changes in fatty acid (FA) oxidation pathway activity. (*B*) PGC-1α pathway status best predicted perioperative changes in cardiac index (CI), whereas DNA-damage signaling best predicted postoperative CI (*C*). Granulocyte colony-stimulating factor (G-CSF) survival signaling best predicted postoperative early diastolic (ED) function measured at the septum (*D*), the mitral annulus (*E*), and the lateral wall (*F*). PROP = propofol patients; SEVO = sevoflurane patients.

acts as a tumor suppressor) checkpoint recently shown to be important in ischemia–reperfusion injury in patients undergoing cardiac surgery.[32] In contrast, the G-CSF survival pathway may have a direct causal role in cardiac fuel selection by regulating STAT3-mediated insulin sensitivity in the heart.[33] Representative members of this pathway are JAK2/3, STAT3, vascular endothelial growth factor, and protein kinase B or Akt, which promote cell survival and angiogenesis.[34,35] STAT3 is known to elicit early preconditioning[36] but also has an obligatory role in the context of late preconditioning.[37] Interestingly, changes in left ventricular diastolic function were best predicted by G-CSF survival pathway activity. The diastolic phase of the cardiac cycle imposes the highest energetic demand on the heart, and thus may be particularly sensitive to ischemia but also receptive to protection by this pathway. G-CSF further exerts antiinflammatory actions,[38] which is consistent with our finding that PAPP-A, a marker of coronary plaque inflammation and instability,[39] was not elevated postoperatively in sevoflurane-treated patients. Finally, G-CSF has positive effects on the mobilization and cardiac homing of bone marrow stem cells, which could accelerate postoperative endothelial and myocardial recovery.[40,41] Recent clinical studies emphasize the important role of PAPP-A in the detection of plaque rupture in patients with acute coronary syndrome, even in the absence of biomarkers of necrosis, potentially identifying high-risk patients whose plaque instability might have been otherwise undetected.[42] PAPP-A was also identified as an independent predictor of future cardiovascular events and of the need for future angioplasty or CABG surgery.[43]

Study Limitations

This study is an observational study and does not allow ultimate interpretation of the causal relations. Some of the observed immediate beneficial effects by sevoflurane may be due to direct signaling events related to preconditioning ("first window"), whereas other more medium- and long-term beneficial effects occurring within hours or days after exposure to sevoflurane may be well—at least in part—explained by genomic reprogramming. Our study can not distinguish between these possibilities, but associates the transcriptional changes to functional and biochemical outcomes. The current study was powered to detect differences in postoperative NT-proBNP levels between the SEVO and PROP groups but not to detect differences in clinical outcome measures. Although the DNA microarray technology has limitations with respect to sensitivity, accuracy, specificity, and reproducibility of results, the currently available data suggest that for abundant transcripts the existence and direction of expression changes can be reliably detected by this method.[44] Given the small number of samples compared with the high number of probes on the microarray (tens of thousands), the uncertainty about the statistical power in microarray analysis remains a limitation of large-scale transcriptional profiling. We have used state-of-the-art bioinformatics tools to address these concerns, particularly with respect to correction for multiple comparisons. Nonetheless, our results should be tested in randomized controlled clinical trials. This study is a short-term assessment of myocardial gene expression. However, we have previously shown in patients undergoing on-pump CABG surgery that favorable short-term changes in gene expression elicited by sevoflurane were associated with improved long-term cardiovascular outcome.[6] Although we carefully selected the patients, there always remains a high biologic variability in human studies compared with well-controlled animal experiments. In particular, medication could have affected myocardial substrate metabolism and biased our findings. In fact, sevoflurane patients received less remifentanil, a preconditioning-mimicking opioid, than propofol patients.[45] However, this should have favored cardiac protection in the propofol patients. Furthermore, despite proper randomization and selection of the patients, our analysis showed that the PGC-1α pathway was differentially regulated at baseline between groups. Therefore, we included this pathway in the multivariate analysis. Despite these caveats, we were able to detect robust functional patterns extending beyond this variability. A relatively healthy population of cardiac surgical patients was selected for the study, which might limit the general application of our findings. End-tidal sevoflurane concentrations were measured, whereas propofol concentrations in the blood were not determined, which might complicate the direct comparison between the two anesthetics. However, sevoflurane and propofol were administered in clinically relevant doses to maintain blood pressure and heart rate within 20% of baseline values. The implications of comparing a lipid-based anesthetic (propofol dissolved in Intralipid) with a lipid-free anesthetic on myocardial energy metabolism are unclear. It could be speculated that the lipid component of propofol but not the anesthetic itself might be responsible for some of the observed differences in cardiac energy metabolism. However, most studies showed that the effects of propofol on cardioprotection are independent of the solvent used.[46] Also, experimental data in mice provide evidence that volatile anesthetics *per se* reduce fatty acid oxidation in the heart.[22] Messenger RNA levels do not always directly translate into changes in protein or functional levels, because additional regulatory mechanisms on the posttranslational level exist. However, the gene expression profile underlying the cellular protein contents of enzymes and transporters is an important determinant of the metabolic phenotype. Finally, for ethical reasons, we used human atrial samples rather than ventricular biopsies to examine gene expression changes. Although there are clearly many disparities (but also many similarities) between human atrial and ventricular tissue, it remains elusive whether the gene expression results from atrial samples in this study can be directly extrapolated to left ventricular tissue. However,

one study found that only approximately 1% of genes, predominantly related to contractile function, were differentially expressed between atrium and left ventricle.[47] Using the Affymetrix chips, Barth *et al.*[48] reported a predominance of gene expression related to energy metabolism and oxidative phosphorylation in ventricles, whereas atrial tissue was prominently expressing transcripts related to signal transduction pathways, which might be specifically sensitive to changes in hemodynamic load and neuroendocrine activity. Of note, in the latter study, significant transcriptional differences were also reported between different regions within the same ventricle, and some chamber-specific markers such as the atrial natriuretic factor were also induced in ventricular tissues. Finally, using Affymetrix U95Av2 arrays, Ohki-Kaneda *et al.*[49] were able to predict left ventricular ejection fraction from right atrial gene expression patterns, implying that the atrial transcriptional profiles of our study are likely of clinical significance with respect to left ventricular gene expression and function.

Conclusions

Considerable effort has been focused on improving perioperative outcome in high-risk surgical patients. In a recent landmark article discussing the need for translation of basic science into clinical practice, anesthetic gases were proposed as readily available model drugs to successfully reproduce cardioprotection in patients.[50] Our data clearly support this view and provide a strong rationale for anesthetic gases in the perioperative management of cardiac surgical patients. In summary, using an integrative physiologic approach, we were able to link anesthetic-induced and constitutive gene regulatory control of myocardial energy metabolism to postoperative cardiac function in patients undergoing off-pump CABG surgery. Our analysis further points to the PGC-1α pathway and the G-CSF survival pathway, integral components of the protective program in the heart, as potential therapeutic targets in perioperative cardioprotection.

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Infarct-remodeled Myocardium Is Receptive to Protection by Isoflurane Postconditioning

Role of Protein Kinase B/Akt Signaling

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Background: **Postinfarct remodeled myocardium exhibits numerous structural and biochemical alterations. So far, it is unknown whether postconditioning elicited by volatile anesthetics can also provide protection in the remodeled myocardium.**

Methods: **Myocardial infarct was induced in male Wistar rats by ligation of the left anterior descending coronary artery. Six weeks later, hearts were buffer-perfused and exposed to 40 min of ischemia followed by 90 min of reperfusion. Anesthetic postconditioning was induced by 15 min of 2.1 vol% isoflurane. In some experiments, LY294002 (15 μM), a phosphatidylinositol 3-kinase inhibitor, was coadministered with isoflurane. Masson's trichrome staining, immunohistochemistry, Western blot analysis, and reverse-transcription polymerase chain reaction served to confirm remodeling. In buffer-perfused hearts, functional recovery was recorded, and acute infarct size was measured using 1% triphenyltetrazolium chloride staining and lactate dehydrogenase release during reperfusion. Western blot analysis was used to determine phosphorylation of reperfusion injury salvage kinases including protein kinase B/Akt and its downstream targets after 15 min of reperfusion.**

Results: **Infarct hearts exhibited typical macroscopic and molecular changes of remodeling. Isoflurane postconditioning improved functional recovery and decreased acute infarct size, as determined by triphenyltetrazolium (35 ± 5% in unprotected hearts *vs.* 8 ± 3% in anesthetic postconditioning; $P < 0.05$) and lactate dehydrogenase release. This protection was abolished by LY294002, which inhibited phosphorylation of protein kinase B/Akt and its downstream targets glycogen synthase kinase 3β, endothelial nitric oxide synthase, and p70S6 kinase.**

Conclusions: **Infarct-remodeled myocardium is receptive to protection by isoflurane postconditioning *via* protein kinase B/Akt signaling. This is the first time to demonstrate that anesthetic postconditioning retains its marked protection in diseased myocardium.**

REMODELING is a maladaptive response to chronic hemodynamic sequelae occurring after large myocardial infarcts and leading to left ventricular dilatation and compensatory hypertrophy of the residual intact cardiac tissue. Apoptosis and fibrosis participate in this chronic process, which has a high propensity for arrhythmogenesis, including sudden cardiac death, and ultimately leads to congestive heart failure.[1,2] In remodeled hearts, left ventricular dysfunction does not only occur in infarcted cicatrized areas, but also occurs in residual intact myocardium. Beside many dramatic macroscopic and microscopic structural changes associated with remodeling, significant alterations in metabolism were previously reported, including a shift toward anaerobic metabolism and a depletion in high-energy phosphates putting the diseased myocardium at particular risk for further ischemic injury.[3]

Postconditioning by volatile anesthetics ("anesthetic postconditioning") is a most effective therapeutic strategy of reducing infarct size after prolonged ischemia. Similar to ischemic postconditioning,[4,5] anesthetic postconditioning enhances the activity of the reperfusion injury salvage kinase (RISK) protein kinase B (PKB)/Akt at the time of reperfusion,[6–8] thereby preventing mitochondrial permeability transition through inhibition of glycogen synthase kinase 3β (GSK3β) and reducing infarct size.[7] Because the onset of reperfusion is predictable and under the control of the operator, this novel therapeutic strategy is particularly promising for the clinical setting.

To date, all experimental studies have evaluated the phenomenon of cardiac postconditioning in healthy juvenile hearts. However, this is far from clinical reality, because diseased myocardium would benefit most from protection by postconditioning. On the other side, some clinical[9,10] and experimental[11] studies provide evidence that diseased myocardium may be less amenable to protection by preconditioning, the most powerful endogenous protective mechanism, which is at the opposite site of ischemia but shares many signaling steps with postconditioning.[12] Therefore, we tested the hypothesis whether protection by

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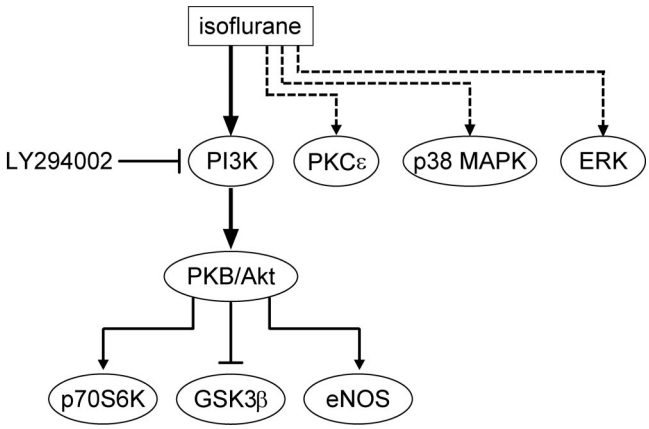

Fig. 1. Investigated signaling components in isoflurane postconditioning in the infarct-remodeled rat heart. Protein kinase B (PKB)/Akt is downstream of phosphatidylinositol 3-kinase (PI3K), a key enzyme in the reperfusion injury salvage kinase cascade (*full arrows*). Phosphorylated PKB/Akt activates 70-kd ribosomal protein S6 kinase (p70S6K) and endothelial nitric oxide synthase (eNOS) and inactivates glycogen synthase kinase 3β (GSK3β) by phosphorylation. Activation of protein kinase C ε (PKC-ε), p38 mitogen-activated protein kinase (p38 MAPK), and extracellular signal–regulated protein kinases (ERKs), potentially involved in cardioprotection from reperfusion injury (*dashed arrows*), were also assessed. LY294002: specific inhibitor of PI3K.

pharmacologic postconditioning elicited by isoflurane would be diminished or lost in markedly remodeled postinfarct myocardium. Additional experiments served to delineate the specific role of PKB/Akt among other RISK components in the protection by isoflurane postconditioning (fig. 1).

The data provided in this study show for the first time that protection by anesthetic postconditioning is fully preserved in postinfarct remodeled myocardium. They further underscore the unique role of PKB/Akt in the cardioprotection by isoflurane postconditioning in the severely diseased heart.

Materials and Methods

This study was conducted in accordance with the guidelines of the Animal Care and Use Committee of the University of Zurich, Zurich, Switzerland.

Induction of Myocardial Infarct In Vivo *to Promote Cardiac Remodeling*

Infarct or sham operations were performed in 200-g male Wistar rats (8–9 weeks old) kept in a 12-h light–dark cycle on commercial rat chow and water *ad libitum*. Ligation of the left anterior descending coronary artery was performed in the intubated rats during isoflurane anesthesia, as previously described.[13] Briefly, the fourth intercostal space was opened, the heart was exteriorized, and the pericardium was cut. The left anterior descending coronary artery was ligated between the left atrium and the pulmonary outflow tract using a 6.0 silk suture. Successful ligation was verified by regional cyanosis of the myocardial surface and ischemic ST-segment changes in the electrocardiogram. The heart was replaced into the thoracic cavity, which was drained from remaining air, and the chest was immediately closed. At the end of operation, all animals received 0.02 mg/kg subcutaneous buprenorphine for postoperative analgesia (with subsequent doses every 12 h for the first 3 postoperative days), and 10 mg/kg subcutaneous enrofloxacin as an antibiotic prophylaxis. A microchip was implanted subcutaneously for identification. Sham-operated animals underwent the same procedure except that the suture was passed under the coronary artery without ligation. A series of separate experiments served to verify the occurrence of an effective remodeling process 6 weeks after ligation. Because successful remodeling and impairment of left ventricular function can be expected only if infarct size reaches approximately 30% of the left ventricular mass,[13,14] acute infarct size was determined 12 h after coronary artery ligation using 1% 2,3,5-triphenyltetrazolium chloride (n = 5). In additional experiments, sham-operated hearts and infarcted hearts were evaluated for their function on the Langendorff apparatus, as well as for morphologic changes and biochemical markers of remodeling on the messenger RNA (mRNA) and protein level (n = 5).

Langendorff Preparation of Rat Hearts and Experimental Protocols

Six weeks after ligation of the left anterior descending coronary artery, rats were heparinized (500 U intraperitoneal) and 15 min later decapitated without previous anesthesia. Hearts were removed and perfused in a noncirculating Langendorff apparatus with Krebs-Henseleit buffer (155 mM Na^+, 5.6 mM K^+, 138 mM Cl^-, 2.1 mM Ca^{2+}, 1.2 mM PO_4^{3-}, 25 mM HCO_{3-}, 0.56 mM Mg^{2+}, 11 mM glucose) gassed with 95% O_2–5% CO_2 (pH 7.4, temperature 37°C). Perfusion pressure was set to 80 mmHg, and left ventricular end-diastolic pressure was set at 10 mmHg after equilibration. Left ventricular developed pressure and derivatives (±dp/dt), left ventricular end-diastolic pressure, epicardial electrocardiogram, coronary flow, and perfusion pressure were recorded, as previously described.[15] After equilibration, spontaneously beating hearts were exposed to 40 min of global test ischemia (fig. 2). Anesthetic postconditioning was induced by isoflurane administered for 15 min at 1.5 minimum alveolar concentration (MAC; 2.1 vol%) right at the onset of reperfusion. The buffer was equilibrated with isoflurane using an Isotec 3 vaporizer (Datex-Ohmeda, Tewksbury, MA) with an air bubbler. Isoflurane concentrations were also measured in the buffer before entering the aortic cannula using a gas chromatograph (Perkin-Elmer, Norwalk, CT): 0.50 ± 0.04 mM. In the blocker experiments, 15 μM of the phosphatidylinositol 3-kinase (PI3K) LY294002 (Alexis, Lausen, Switzerland),

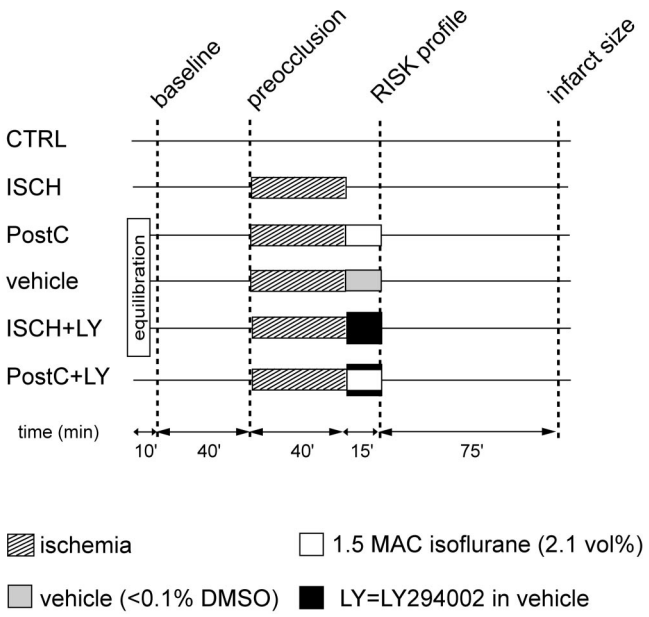

Fig. 2. Treatment protocols. Anesthetic postconditioning (PostC) = isoflurane administered for 15 min (1.5 minimum alveolar concentration [MAC]) right at the onset of reperfusion after 40 min of test ischemia; CTRL = time-matched perfusion of remodeled hearts; ISCH = unprotected remodeled hearts exposed to 40 min of ischemia alone; LY = LY294002. LY294002 dissolved in dimethyl sulfoxide (DMSO; < 0.1%) was coadministered with isoflurane during early reperfusion. Reperfusion injury salvage kinases (RISKs) were assayed after 15 min of reperfusion, and infarct size was determined after 90 min of reperfusion. Five hearts were used in each group.

dissolved in dimethyl sulfoxide at a final concentration of less than 0.1%, was coadministered with isoflurane or given alone during the first 15 min of reperfusion.[5,7] Dimethyl sulfoxide alone did not affect functional recovery, infarct size, or the phosphorylation status of any of the measured kinases. Hearts subjected to ischemia and reperfusion alone served as ischemic control. For each experimental group, five hearts (n = 5) were prepared, and functional parameters were recorded (fig. 2). To characterize myocardial function 6 weeks after induction of infarct, additional Langendorff perfusion studies were performed as described to asses cardiac function *ex vivo* in infarct-remodeled and sham-operated hearts (n = 5 in each group).

Determination of Infarct Size and Myocardial Damage

Infarct size was determined by 2,3,5-triphenyltetrazolium chloride staining after 90 min of reperfusion. Briefly, hearts were frozen at −20°C for 2 h at the end of the experiment and sliced into five 2-mm cross sections. The sections were incubated at 37°C for 30 min in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M phosphate buffer (pH = 7.4), fixed in 10% formaldehyde, and digitally photographed. Planimetric analysis was performed using ImageJ 1.33.§§ Briefly, the chronic infarct (bright white) was subtracted from the total left ventricular slice area to obtain the area at risk. Infarct size was determined by dividing the freshly necrotic area (salmon pink) of the left ventricle by the area at risk. Therefore, areas infarcted *in vivo* by coronary ligation (chronic infarct) were excluded from subsequent infarct size analysis. In addition, myocardial damage was estimated by measuring the release of lactate dehydrogenase (LDH) from necrotic tissue. For this purpose, the perfusate was collected, and LDH was determined by the Roche/Hitachi 917 (sensitivity 6 U/l, intraassay and interassay coefficients of variance < 1%; Hitachi Corp., Tokyo, Japan).

Western Blot Analysis

Separate experiments served to determine the activities of kinases after 15 min of reperfusion (n = 5 in each group; fig. 1). The following antibodies were used for Western blot analysis: polyclonal antibody specific for atrial natriuretic peptide and protein kinase C ε (PKC-ε) (Santa Cruz, Biotechnology, Inc., Santa Cruz, CA); polyclonal antibodies specific for phospho-PKB/Akt (Ser-473), GSK3β, phospho-GSK3β (Ser-9), p70S6 kinase (p70S6K), phospho-p70S6K (Thr-389), extracellular signal–regulated kinase1/2 (ERK1/2), phospho-ERK1/2 (Thr-202/Tyr-204), p38 mitogen-activated protein kinase, phospho-p38 mitogen-activated protein kinase (Thr-180/Tyr-182), endothelial nitric oxide synthase (eNOS), and phospho-eNOS (Ser-1177) (Cell Signaling Technology, Beverly, CA); polyclonal phospho-PKC-ε (Ser-729) antibody (Upstate, Milton Keynes, United Kingdom); monoclonal anti–pan-actin (Chemicon, Temecula, CA); monoclonal anti–α-tubulin (Sigma, St. Louis, MO). Monoclonal antibodies against myosin heavy chain α and β (α- and β-MHC) were a gift from Simon M. Hughes, Ph.D. (Division of Cell and Molecular Biology, The Randall Institute, King's College London, London, United Kingdom). Polyclonal anti-PKB/Akt antibody was a gift from Brian A. Hemmings, Ph.D. (Friedrich Miescher Institute, Basel, Switzerland). Polyclonal antibody against α-skeletal actin was a gift from Christine Chapponier, Ph.D. (Department of Pathology, University of Geneva, Geneva, Switzerland). After 15 min of reperfusion, left ventricular tissue at risk was taken and frozen in liquid nitrogen. Subsequently, it was powdered and homogenized in lysis buffer containing 62.5 mM Tris-HCl at pH 6.8, 2% sodium dodecyl sulfate, 4 mM EDTA, and 7% sucrose. Extracts were boiled at 95°C for 5 min followed by 30 min centrifugation at 12,000*g*. Protein concentrations in the supernatants were determined by the Bradford method. Extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene diflouride membranes (Amersham Biosciences, Buckinghamshire, United Kingdom). Membranes were blocked with phosphate-buffered saline (PBS) containing 5% nonfat dry milk and 0.05%

§§ http://rsb.info.nih.gov/ij/. Accessed December 1, 2004.

Tween-20 for 1 h and then incubated with the primary antibody. The membranes were washed and incubated with horseradish peroxidase–conjugated goat antirabbit immunoglobulin G (Pierce, Rockford, IL). After extensive washing, the blots were exposed to films (Fuji Photo Film Co., Kanakawa, Japan) for 10 s up to 1 min to obtain a linear response by the enhanced chemiluminescence method (Pierce, Rockford, IL). The quantity of the immunoreactive bands was determined by densitometry using ImageJ 1.33.§§

mRNA Extraction and Real-time Quantitative Polymerase Chain Reaction

Total RNA was prepared using an RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland). First strand complementary DNA (cDNA) was synthesized from 1.0 μg total RNA using SuperScript II reverse transcriptase (Invitrogen, Basel, Switzerland) and oligo-dT as a primer. Real-time polymerase chain reaction was accomplished with the specific primers at an annealing temperature of 55°C on a Stratagene MX3000 real-time sequence detection instrument using the Brilliant SYBR Green QPCR kit (Stratagene Europe, Amsterdam, The Netherlands). The following primers were used: atrial natriuretic peptide: 5′-ATCACCAAGGGCTTCTTCCT-3′ (sense) and 5′-TGTTGGACACCGCACTGTAT-3′ (antisense); brain natriuretic peptide: 5′-GGAAATGGCTCAGAGACAGC-3′ (sense) and 5′-CGGTCTATCTTCTGCCCAAA-3′ (antisense); β-MHC: 5′-TCTTGTGCTACCCAACCCTAA-3′ (sense) and 5′-TTGGCTTGAAGGAAAATTGC-3′; α-MHC: 5′-GCAGAAAATGCACGATG-3′ (sense) and 5′-TACAGGCAAAGTCAAGC-3′ (antisense); α-skeletal-actin: 5′-CACGGCATTATCACCAACTG-3′ (sense) and 5′-CCGGAGGCATAGAGAGACAG-3′ (antisense). The expression levels were measured in triplicates of the first strand cDNA, and α-tubulin was used for normalization of the data. The sense and antisense primers for α-tubulin were 5′-CCATGCGTGAGTGTATCTCC-3′ and 5′-GTGCCAGTGCGAACTTCATC-3′, respectively.

Histologic Assessment

Left ventricular tissue samples were placed in optimal cutting temperature medium (Tissue-Tek; Sakura Finetek Inc., Torrance, CA), frozen in liquid nitrogen, and stored at −70°C. Cryosections (5 μm) were collected on gelatin-precoated slides and air dried. The sections were fixed in precooled methanol at −20°C for 10 min. After washing with PBS, the sections were blocked with 5% normal goat serum at room temperature for 1 h. The sections were incubated at 4°C overnight with the following primary antibodies in 1% normal goat serum and PBS: mouse monoclonal anti–N-cadherin (Zymed, Basel, Switzerland), mouse monoclonal antidesmin (DakoCytomation AG, Baar, Switzerland), mouse monoclonal anti–α-MHC and anti–β-MHC (a gift from Simon M. Hughes, Ph.D., The Randall Institute, King's College London, United Kingdom). Subsequently, the sections were washed in PBS and incubated with Alexa Fluor-488–labeled anti-mouse immunoglobulin G (Molecular Probes, Eugene, OR) and 4′-6-diamidino-2-phenylindole (DAPI, 10 ng/ml; Sigma-Aldrich, Buchs, Switzerland) in 1% normal goat serum with PBS at room temperature in the dark for 1 h. After several steps of washing, the sections were mounted in Lisbeth's mounting medium (33 mM Tris, pH 9.5, 70% glycerol, 50 mg/ml n-propyl gallate) and examined using an epifluorescence microscope Axiovert M200 (Zeiss, Jena, Germany). Additional conventional histologic staining including hematoxylin–eosin and Masson's trichrome was performed. Hearts were perfused with 4% paraformaldehyde and embedded in paraffin. Sections of 3 μm were cut, mounted, and stained.

Statistics

All data are presented as mean ± SD. For hemodynamic data and LDH release, repeated-measures analysis of variance was used to evaluate differences over time between groups. An unpaired *t* test was used to compare groups at identical time points, and a paired *t* test was used to compare within groups over time. *P* values were multiplied by the number of comparisons that were made (Bonferroni correction). For all other data, one-way analysis of variance with *post hoc* Tukey test was used for multiple comparisons. $P < 0.05$ was considered significant. SigmaStat (version 2.0; SPSS Science, Chicago, IL) was used for analysis.

Results

Postinfarct Remodeled Hearts Exhibit Characteristic Macroscopic and Ultrastructural Changes

Ligation of the left anterior descending coronary artery induced extended infarcts (35 ± 5%), which were sufficient to initiate and promote myocardial remodeling in

Table 1. Characteristics of Sham-operated and Infarcted Hearts

	Sham Rats	Infarct Rats
Body weight, g*	296 ± 17	288 ± 20
Heart weight (wet), g*	1.21 ± 0.07	1.66 ± 0.11‡
Heart weight/body weight, g/kg	4.07 ± 0.37	5.70 ± 0.56‡
Infarct size, % of left ventricular wall	—	35 ± 5†
Heart rate, beats/min	302 ± 13	294 ± 10
LVDP, mmHg	100 ± 6	73 ± 8‡
Coronary flow, ml/min	14.5 ± 1.0	15.0 ± 0.8

Baseline hemodynamics were determined on the Langendorff apparatus 20 min after initiating buffer perfusion. Cardiac function was measured under similar conditions of 10 mmHg left ventricular end-diastolic pressure.

* Body and heart weights as determined 6 weeks after ligation of the left anterior descending coronary artery (n = 5). † Individual infarct sizes were as follows: heart 1 = 41%; heart 2 = 32%; heart 3 = 37%; heart 4 = 28%; heart 5 = 36%. ‡ $P < 0.05$.

LVDP = left ventricular developed pressure.

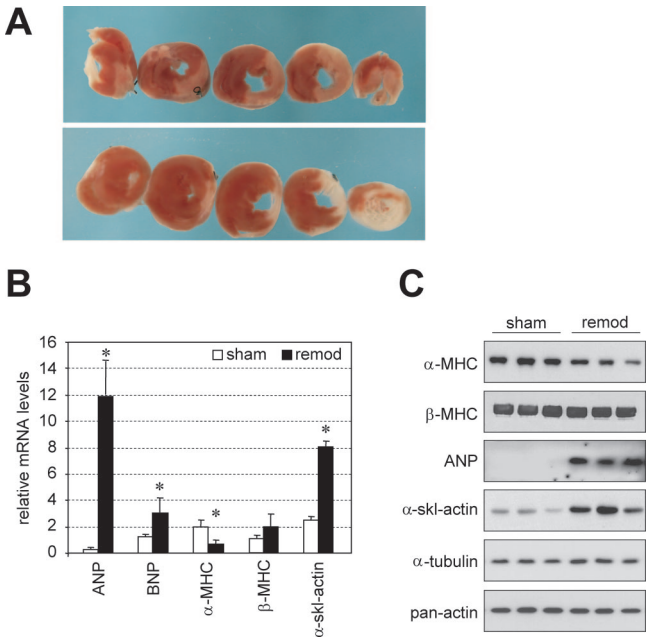

Fig. 3. Cardiac remodeling. Left anterior descending coronary artery was ligated, and infarct size was determined after 12 h using 1% triphenyltetrazolium chloride staining (*A*, *upper panel*). Viable myocardium is stained *brick-stone red*, whereas freshly infarcted myocardium is stained *salmon pink*. Note the *black ties* on the two basal transverse heart sections. A mean infarct size of 35 ± 5% was obtained (see also table 1). Six weeks after ligation, necrosis was replaced by scar tissue, and compensatory hypertrophy developed (*A*, *lower panel*). Chronic infarct is stained *bright white*. Transcript levels of remodeling markers (*B*): atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), α- and β-myosin heavy chains (α- and β-MHC), α-skeletal-actin (α-skl-actin). Western blot analysis of remodeling markers (*C*): ANP, BNP, α- and β-MHC, α-skl-actin. Alpha-tubulin and total actin (pan-actin) were used as loading controls. Remod = postinfarct remodeled hearts; sham = sham-operated hearts. Data are given as mean ± SD (n = 5 in each group). * *P* < 0.05 *versus* sham.

the postinfarct healing period (table 1). The ratio of heart weight over body weight was significantly increased 6 weeks after ligation in the infarcted hearts (table 1) compared with sham-operated hearts, indicating compensatory hypertrophy, predominantly in the septal area of the heart (fig. 3A). Of note, heart weight (1.63 ± 0.13 g) and heart weight/body weight ratio (5.80 ± 0.33 g/kg) were also markedly increased in all animals used for experimentation. Consistent with loss of viable myocardium and hypertrophy was the observed reduced contractile function of the remodeled hearts as determined *ex vivo* on the Langendorff apparatus under control conditions (left ventricular diastolic pressure set at 10 mmHg) (table 1). At the mRNA level, transcript levels for atrial natriuretic peptide, brain natriuretic peptide, and α-skeletal-actin were increased, whereas the transcript for α-MHC was nearly absent (fig. 3B). Similarly, at the protein level, atrial natriuretic peptide and α-skeletal-actin were overexpressed, and α-MHC was markedly reduced (fig. 3C). Masson's trichrome staining of whole heart longitudinal and transverse sections exhibited increased amounts of collagen in the chronic infarct scar and compensatory hypertrophy with dilated spherical left ventricular cavities (fig. 4). Detailed immunohistochemical analysis including markers of cell–cell interaction (N-cadherin), contractile filaments α- and β-MHC, and extracellular matrix (desmin) revealed differences between sham-operated and infarcted hearts (fig. 4). Comparable to previous studies with similar models,[16–18] there was no difference in the phosphorylation status of PKB/Akt and ERK1/2 between sham-operated and infarcted hearts 6 weeks after operation (data not shown). Together, the results of these experiments demonstrate significant morphologic changes at the macroscopic and microscopic levels in chronically remodeled postinfarct myocardium.

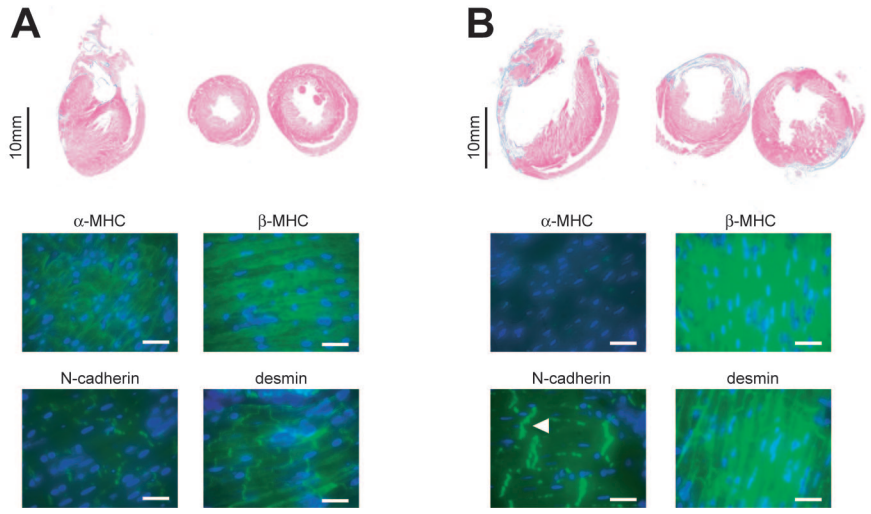

Fig. 4. Histochemistry of remodeled myocardium. Masson's trichrome staining and immunohistochemical stainings for α- and β-myosin heavy chain (α- and β-MHC), N-cadherin, and desmin were used to visualize macroscopic and ultrastructural changes in remodeled hearts. Representative sham-operated hearts 6 weeks after operation are shown on the *left* (*A*), and postinfarct remodeled hearts 6 weeks after ligation are shown on the *right* (*B*). Note the spherical shape of the remodeled hearts with infarct wall thinning and ventricular dilatation in contrast to the ellipsoid shape of the sham-operated hearts. Transverse sections were prepared from tissue taken at the midpapillary level. The fluorescence intensity was increased in the remodeled myocardium for β-MHC, N-cadherin, and desmin but markedly reduced for α-MHC. N-cadherin was accumulated at intercalated disks (*arrowhead*) in remodeled myocardium. *Bar* represents distance of 50 μm.

Table 2. Hemodynamics

			Reperfusion		
	Baseline	Preocclusion Value‡	15 min	30 min	90 min
LVDP, mmHg					
CTRL	69 ± 7	71 ± 8	70 ± 8	72 ± 10	73 ± 10
ISCH	68 ± 6	73 ± 9	6 ± 3*†	17 ± 4*†	16 ± 3*†
ISCH + LY	65 ± 8	70 ± 12	7 ± 2*†	15 ± 3*†	17 ± 4*†
PostC	67 ± 5	69 ± 8	21 ± 4*†	53 ± 7*†	56 ± 4*†
PostC + LY	70 ± 9	72 ± 5	5 ± 2*†	16 ± 4*†	15 ± 4*†
DMSO	65 ± 10	68 ± 4	7 ± 3*†	18 ± 5*†	14 ± 3*†
LVEDP, mmHg					
CTRL	9 ± 2	8 ± 1	9 ± 2	10 ± 1	9 ± 1
ISCH	8 ± 2	9 ± 2	42 ± 9*†	47 ± 7*†	39 ± 8*†
ISCH + LY	9 ± 1	10 ± 3	40 ± 8*†	45 ± 5*†	39 ± 6*†
PostC	8 ± 1	9 ± 2	19 ± 5*†	19 ± 1*†	16 ± 2*†
PostC + LY	10 ± 2	8 ± 1	41 ± 6*†	41 ± 8*†	39 ± 7*†
DMSO	10 ± 1	10 ± 3	42 ± 8*†	41 ± 3*†	40 ± 5*†
+dp/dt, mmHg/s					
CTRL	2,060 ± 200	2,100 ± 159	2,080 ± 220	2,070 ± 190	1,880 ± 210
ISCH	1,900 ± 300	2,076 ± 200	160 ± 100*†	570 ± 320*†	720 ± 200*†
ISCH + LY	1,867 ± 240	1,900 ± 230	130 ± 50*†	550 ± 290*†	800 ± 250*†
PostC	2,160 ± 181	2,190 ± 200	460 ± 100*†	1,260 ± 200*†	1,588 ± 75*†
PostC + LY	1,936 ± 307	2,076 ± 280	1,650 ± 100*†	600 ± 300*†	720 ± 250*†
DMSO	1,960 ± 305	2,070 ± 282	130 ± 100*†	550 ± 310*†	700 ± 240*†
−dp/dt, mmHg/s					
CTRL	1,540 ± 114	1,400 ± 234	1,454 ± 160	1,400 ± 100	1,580 ± 130
ISCH	1,600 ± 310	1,520 ± 400	80 ± 30*†	224 ± 123*†	264 ± 112*†
ISCH + LY	1,650 ± 400	1,440 ± 170	80 ± 25*†	180 ± 95*†	260 ± 200*†
PostC	1,670 ± 300	1,480 ± 200	350 ± 120*†	800 ± 120*†	1,070 ± 205*†
PostC + LY	1,600 ± 400	1,700 ± 400	85 ± 40*†	214 ± 100*†	274 ± 100*†
DMSO	1,400 ± 210	1,580 ± 420	62 ± 40*†	234 ± 120*†	294 ± 111*†
CF, ml/min					
CTRL	13.0 ± 1.3	13.4 ± 2.3	12.0 ± 1.4	12.6 ± 1.1	12.5 ± 1.5
ISCH	12.0 ± 1.5	11.6 ± 1.5	2.8 ± 1.6*†	3.4 ± 1.1*†	3.8 ± 0.4*†
ISCH + LY	12.8 ± 0.8	12.0 ± 1.8	2.4 ± 1.1*†	2.6 ± 1.5*†	3.6 ± 1.6*†
PostC	13.4 ± 2.0	12.2 ± 2.0	9.8 ± 1.3*†	10.6 ± 1.1*†	11.2 ± 0.8*†
PostC + LY	13.6 ± 1.7	12.8 ± 0.8	2.2 ± 0.8*†	3.4 ± 1.2*†	2.6 ± 0.8*†
DMSO	12.8 ± 1.0	12.4 ± 2.1	2.4 ± 1.1*†	2.8 ± 1.3*†	3.0 ± 1.8*†
HR, beats/min					
CTRL	283 ± 6	306 ± 19	300 ± 23	285 ± 20	300 ± 18
ISCH	300 ± 25	292 ± 16	180 ± 60*†	192 ± 27*†	204 ± 21*†
ISCH + LY	296 ± 11	294 ± 15	134 ± 50*†	150 ± 33*†	196 ± 30*†
PostC	296 ± 20	296 ± 11	192 ± 18*†	246 ± 12*†	243 ± 11*†
PostC + LY	282 ± 15	302 ± 21	136 ± 33*†	196 ± 20*†	204 ± 13*†
DMSO	300 ± 27	291 ± 20	138 ± 30*†	190 ± 27*†	210 ± 12*†

Data are presented as mean ± SD (n = 5 for each group).

* Significantly ($P < 0.05$) different from baseline (intragroup comparison). † Significantly ($P < 0.05$) different from respective value in CTRL and ISCH (intergroup comparison). ‡ Before test ischemia.

+dp/dt = inotropy; −dp/dt = lusitropy; CF = coronary flow; CTRL = control (time-matched perfusion of remodeled hearts); DMSO = dimethyl sulfoxide (< 0.1%); HR = heart rate; ISCH = test ischemia without postconditioning; LVDP = left ventricular developed pressure; LVEDP = left ventricular end-diastolic pressure; LY = LY294002; PostC = isoflurane postconditioning.

Protection by Isoflurane Postconditioning Depends on PI3K-PKB/Akt Signaling Pathway in the Remodeled Myocardium

To determine whether isoflurane postconditioning would also provide protection in the diseased remodeled myocardium, chronically infarcted hearts were exposed to 40 min of ischemia. Anesthetic postconditioning with isoflurane (1.5 MAC) administered for 15 min immediately at the onset of reperfusion significantly improved functional recovery and decreased infarct size when compared with unprotected remodeled hearts (table 2 and fig. 5). Protection by isoflurane postconditioning was completely abolished by coadministration of the PI3K inhibitor LY294002. LY294002 (or dimethyl sulfoxide) alone administered during reperfusion did not further deteriorate postischemic recovery, nor did it affect infarct size (table 1 and fig. 5A). To independently corroborate the results of infarct size determinations by triphenyltetrazolium, LDH release was measured in the perfusate during reperfusion. LDH release was significantly reduced by isoflurane postconditioning, and this protection was abolished by LY294002 administration

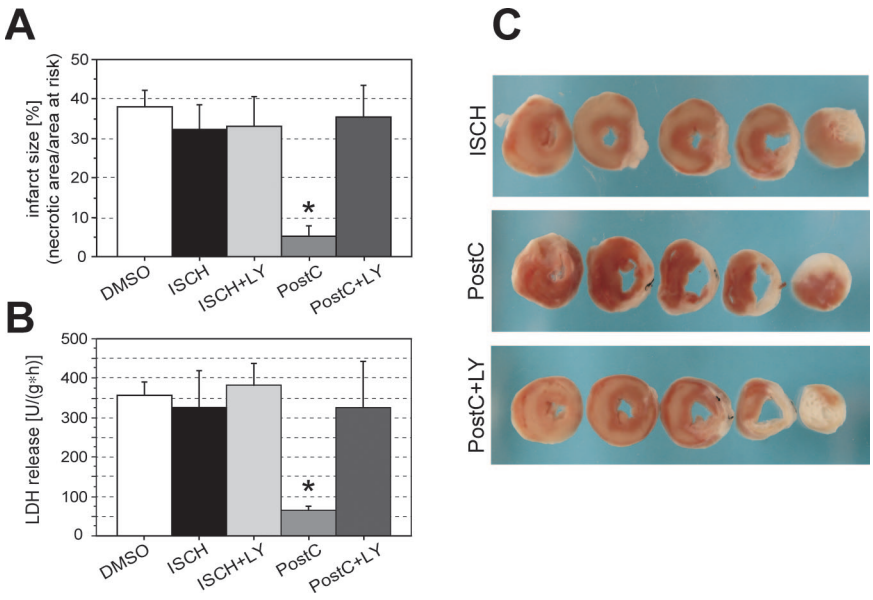

Fig. 5. Infarct size was determined using 1% triphenyltetrazolium chloride staining, as described in the Materials and Methods section. Areas infarcted *in vivo* by coronary artery ligation were excluded from infarct size determination (*A*). The scarred chronic infarct (*white*) resulting from coronary ligation was clearly distinguished from fresh infarcts (*salmon pink*). Release of lactate dehydrogenase (LDH) during reperfusion (*B*) served as an independent method to estimate infarct size (see Materials and Methods). Transverse sections of representative experiments (*C*). DMSO = dimethyl sulfoxide (< 0.1%; used to dissolve LY294002); ISCH = unprotected remodeled hearts exposed to ischemia–reperfusion alone; LY = LY294002 (15 μM); PostC = anesthetic postconditioning. Data are given as mean ± SD (n = 5 in each group). * $P < 0.05$ *versus* ISCH.

(fig. 5B). These results provide strong evidence that protection by isoflurane postconditioning is preserved and operative in this model of postinfarct myocardium.

Isoflurane Postconditioning Activates PKB/Akt, Which Subsequently Phosphorylates the Downstream Targets GSK3β, eNOS, and p70S6K in a PI3K-dependent Manner

It has been well demonstrated that GSK3β is a major downstream target of PKB/Akt and that phosphorylation of the N-terminal Ser-9 residue by PKB/Akt leads to inhibition of GSK3β.[7] Similar to our previous results in healthy myocardium, we here show that 40 min of test ischemia alone increased phosphorylation of PKB/Akt and GSK3β to a certain extent when compared with time-matched perfusion (figs. 6A and B). However, when isoflurane was administered during the early reperfusion phase, a significant additional increase in phosphorylation of PKB/Akt and GSK3β was observed (figs. 6A and B). Both ischemia-reperfusion–induced and isoflurane-induced phosphorylation of PKB/Akt and GSK3β were strongly suppressed by LY294002, a specific inhibitor of PI3K. In contrast, eNOS and p70S6K, downstream targets of PKB/Akt, were only marginally activated by ischemia–reperfusion alone, but strikingly enhanced by isoflurane administration and completely abolished by LY294002 (figs. 6C and D). LY294002 (or dimethyl sulfoxide) alone administered during reperfusion did not alter phosphorylation of the investigated enzymes when compared with ischemic control (data not shown). Taken together, these experiments show for the first time a PI3K-dependent activation of three important highly protective downstream targets of PKB/Akt including GSK3β, eNOS, and p70S6K in response to isoflurane postconditioning. The data further provide evidence that PKB/Akt signaling is functional in remodeled myocardium.

Profile of Potential RISKs Does Not Reveal Additional Kinases Involved in the Protection by Isoflurane Postconditioning in This Postinfarct Rat Heart Model

In sharp contrast to PKB/Akt and its downstream signaling targets, ischemia-reperfusion–induced activation of ERK1/2 and p38 mitogen-activated protein kinase was not altered by isoflurane. Also, no change was detected in the PKC-ε phosphorylation status of the various experimental protocols, implying that the role of these kinases may be of limited importance in mediating the protection by isoflurane postconditioning (fig. 6E). The data clearly underscore the unique role of PKB/Akt signaling in the protection of the remodeled myocardium by isoflurane postconditioning.

Discussion

Here, we show for the first time that isoflurane postconditioning retains its protection against ischemia in the severely diseased myocardium. Our experimental model of a postinfarct remodeled myocardium exhibited macroscopic and ultrastructural changes consistent with marked architectural rearrangements of the myocardium, which were accompanied with characteristic alterations at the gene and protein expression level. Despite this profound remodeling process, the diseased hearts were still receptive to functional and structural protection by anesthetic postconditioning. Of note, infarct size reductions by isoflurane postconditioning, as measured by triphenyltetrazolium staining, were corroborated by a reduced release of the necrosis marker LDH into the perfusate during reperfusion. Furthermore, the preserved protection in the postinfarct remodeled hearts completely depended on activation of PI3K and was commensurate with enhanced phosphorylation of the

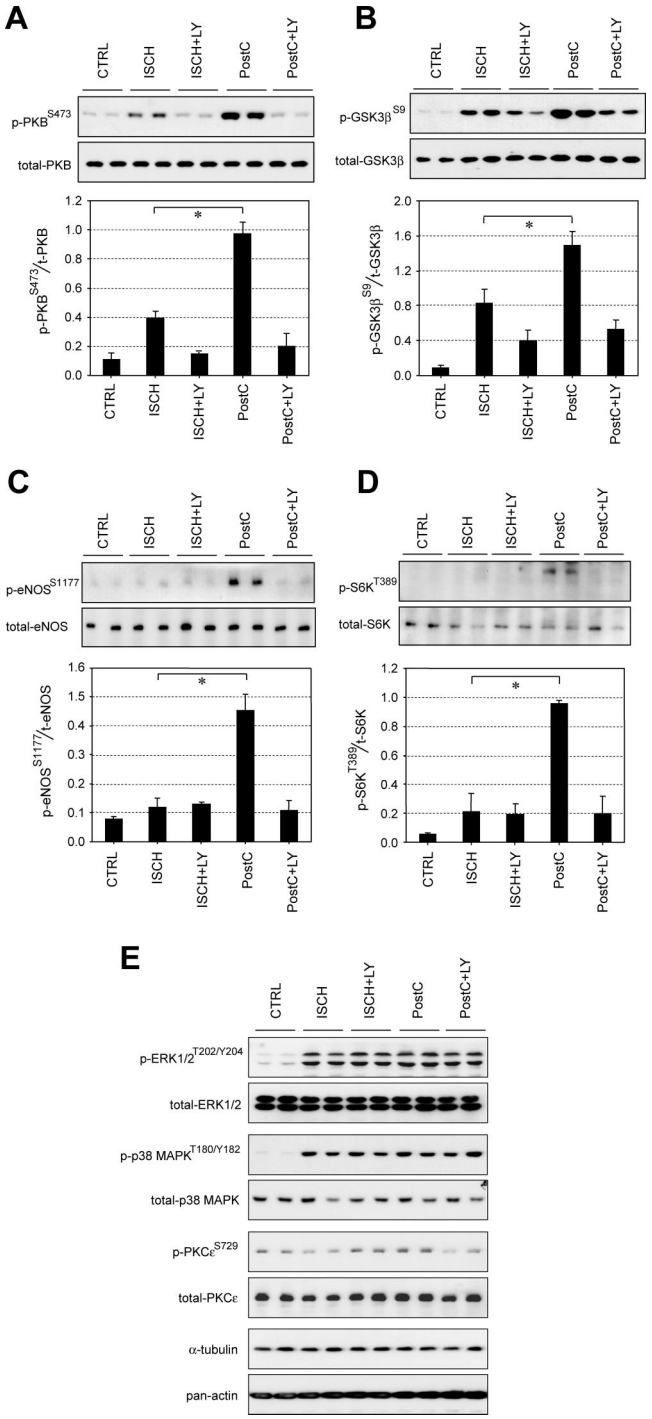

Fig. 6. Western blots analysis. Phosphorylation status of protein kinase B/Akt (PKB, 60 kd, phosphorylation at Ser-473) (*A*), glycogen synthase kinase 3β (GSK3β, 46 kd, phosphorylation at Ser-9) (*B*), endothelial nitric oxide (eNOS, 140 kd, phosphorylation at Ser-1177) (*C*), ribosomal S6 kinase (p70S6K, 70/85 kd, phosphorylation at Thr-389) (*D*), extracellular signal–regulated kinase 1/2 (ERK1/2, 44 and 42 kd, phosphorylation at Thr-202/Tyr-204), p38 mitogen-activated protein kinase (p38 MAPK, 43 kd, phosphorylation at Thr-180/Tyr-182), and protein kinase C ε (PKC-ε, 95 kd, phosphorylation at Ser-729) (*E*) were analyzed with specific phospho-antibodies. CTRL = time-matched perfusion; ISCH = unprotected remodeled hearts exposed to ischemia/reperfusion alone; LY = LY 294002 (15 μM); PostC = anesthetic postconditioning. Data are given as mean ± SD (n = 5 in each group). * $P < 0.05$ PostC *versus* ISCH.

prosurvival PKB/Akt and its protective downstream signaling targets GSK3β, eNOS, and p70S6K. This confirms and further extends previous work[6–8,19] showing the unique role of PKB/Akt signaling in the cellular protection by volatile anesthetics not only in the healthy but also in the diseased heart. Our observation of a functional anesthetic postconditioning mechanism in severely diseased myocardium is of great clinical importance, because an increasing number of elderly patients with hypertension, coronary artery disease, or congestive heart failure and concomitant myocardial remodeling must undergo high-risk cardiac or noncardiac surgery.

Postconditioning: Old Concept Behind a Novel Term?

Postconditioning was first established in 2003 by Vinten-Johansen's group.[20,21] It is a mechanical process whereby brief cycles of alternating ischemia and reperfusion right at the onset of reperfusion markedly decrease ischemic heart damage. Beneficial effects by gradual reperfusion or administration of postconditioning-mimicking agents at the time of reperfusion ("pharmacologic postconditioning") including volatile anesthetics[22] have been known since the 1980s (for review, see Vinten-Johansen *et al.*[23]). However, postconditioning is not simply a passive form of gentle controlled or chemically modified reperfusion, but rather an active biologic process recruiting prosurvival signaling pathways.[12] Yang *et al.*[24] reported a 70% reduction in infarct size using six cycles of 10 s reperfusion–10 s ischemia starting at the beginning of reperfusion in buffer-perfused rabbit hearts, a magnitude of protection similar to the one previously reported for ischemic[25] and anesthetic[26,27] preconditioning. The current study now shows that administration of 1.5 MAC isoflurane during the first 15 min of reperfusion can achieve comparable protection in remodeled hearts.

Cardioprotection in Remodeled Myocardium

Loss of functional myocardium imposes deleterious biomechanical and neurohumoral stress on the residual tissue, leading to compensatory hypertrophy but also additional myocyte death and slippage. This vicious circle, also called remodeling, ultimately results in ventricular dilatation and pump failure. Remodeling is characterized by activation of the fetal gene program and is accompanied by misexpression and rearrangement of myofibrillar, cytoskeletal, and extracellular proteins.[28] Our postinfarct hearts clearly exhibited marked remodeling, with α-skeletal-actin, β-MHC, and desmin being up-regulated. Increased micromechanical stress at cell–cell contacts could be directly visualized by accumulation of N-cadherin at adherens junctions.[29] In accordance with our study, previous experimental data showed that 6 weeks after a large infarction, contractile

dysfunction and hypertrophy consistently evolve in rats.[30] In the clinical setting, angina before myocardial infarction, a clinical correlate to ischemic preconditioning, protects against left ventricular remodelling.[31] Recently, we reported that anesthetic preconditioning but not postconditioning effectively suppressed the early activation of the deleterious remodeling program after ischemia.[27] Vice versa, remodeling may disrupt signaling pathways and abolish innate protective cellular strategies such as preconditioning and postconditioning, and render the myocardium refractory to protection and more susceptible to ischemia. Results from muscle slices of human right atrial appendices of patients with failing hearts indicate that failing myocardium is less amenable to protection by ischemic preconditioning.[11] In fact, loss of preconditioning and postconditioning may be involved in the poor prognosis of patients with postinfarct ventricular remodeling.[32] In line with this observation are findings from a study investigating the effects of preconditioning in postinfarct rabbit hearts, demonstrating complete failure of ischemic but not diazoxide-induced preconditioning to protect the remodeled heart.[33] Refractoriness of the remodeled myocardium to preconditioning was explained by interruption of signal transduction between G-protein and PKC-ε.[34] Other conditions such as hyperglycemia[35] and aging[36] were also found to diminish anesthetic preconditioning. On the other hand, functional preconditioning was reported in severely atherosclerotic ApoE/LDLr$^{-/-}$ knockout mice and several rat heart models of hypertrophied myocardium (for review, see Zaugg *et al.*[37]). Our study now extends these observations and fills a gap showing protection by isoflurane postconditioning in postinfarct remodeled hearts. Whether postconditioning is more effective than preconditioning in the remodeled heart remains to be investigated in future studies.

Signaling in Postconditioning: Current Controversies

Both preconditioning and postconditioning activate RISKs.[38] In ischemic preconditioning, Hausenloy *et al.*[12] previously reported that inhibition of PKB/Akt and ERK1/2 during the first 15 min of reperfusion completely abolished protection. In ischemic postconditioning, Tsang *et al.*[5] and Yang *et al.*[4] recently uncovered the importance of PKB/Akt but also ERK1/2 signaling. Similarly, da Silva *et al.*[39] showed the pivotal role of ERK1/2 during reperfusion in isoflurane preconditioning, while three recent studies unequivocally demonstrate the dependence of isoflurane postconditioning on activation of the PI3K-PKB/Akt pathway.[6–8] However, the relative importance of PKB/Akt and ERK1/2 in mediating the protection of postconditioning is still controversial. A study in isolated perfused rabbit hearts of regional ischemia reports that activation of ERK1/2 but not PKB/Akt is required.[40] Conversely, another study investigating pharmacologic postconditioning by bradykinin suggests that PKB/Akt is upstream of ERK1/2.[41] The authors also found that bradykinin administered at reperfusion caused only a brisk transient increase of ERK1/2 in half of the hearts. These obviously divergent observations may depend on species, the site of tissue sampling (transmural *vs.* epicardial/endocardial), or both but certainly require additional investigation. Consistent with previous reports showing a complex multiphase time course of PKB/Akt and ERK1/2 activation in hypertrophied, ischemic, and failing hearts,[16–18] we could not observe activation of these kinases 6 weeks after coronary ligation when compared with sham-operated hearts.

In addition to RISKs, preconditioning and postconditioning share other signaling components, such as the triggers adenosine and opioids,[8] reactive oxygen species, nitric oxide, and the end-effectors adenine trinucleotide phosphate–dependent potassium channels[42,43] and the mitochondrial permeability transition pore.[7,44] We recently demonstrated that anesthetic postconditioning prevents opening of the mitochondrial permeability transition pore *via* inhibition of GSK3β.[7] The current study now also shows the phosphorylation of the PKB/Akt downstream targets eNOS and p70S6K in anesthetic postconditioning, confirming that most of the critical signaling entities are shared between ischemic and anesthetic postconditioning.[5] Activation of eNOS improves endothelial function, whereas activation of p70S6K facilitates protein synthesis. Taken together, activation of survival signaling by anesthetic postconditioning is preserved in postinfarct remodeled hearts and successfully marshaled by PKB/Akt.

Clinical Implications

Reduction of infarct size is clinically important because it directly correlates with survival. We have previously shown that brief administration of sevoflurane on the fully established cardiopulmonary bypass before induction of cardioplegia significantly improves postoperative cardiac function[45] as well as long-term cardiovascular outcome[46] in patients undergoing coronary artery bypass graft surgery. The concept of postconditioning now shows that reperfusion *per se* is a major factor contributing to ischemic damage and opens the clinically attractive possibility to successfully treat "ischemic" damage at the time of reperfusion. A recent landmark study investigated ischemic postconditioning in patients undergoing coronary angioplasty and stenting for acute myocardial infarction.[47] In this study, creatine kinase release was markedly decreased by 36% in patients treated with four episodes of 1-min balloon inflations starting within 1 min of reflow. Similarly, De Hert *et al.*[48] showed beneficial cardiac effects of anesthetic postconditioning in patients undergoing on-pump coronary artery bypass grafting. Interestingly, in this study the combination of

anesthetic preconditioning and postconditioning was most protective in accordance with a recent *in vivo* animal study[43] and the molecular findings by Lucchinetti *et al.*[27] demonstrating differential but potentially synergistic protective gene expression patterns after anesthetic preconditioning and postconditioning. Because ischemic postconditioning by repetitive clamping or balloon inflation may lead to embolization or endothelial damage, pharmacologic postconditioning by volatile anesthetics should be preferred in the clinical setting. Anesthetic postconditioning of remodeled human hearts may particularly hold promise in nonsurgical coronary artery interventions.

Limitations of the Study and Specific Comments

The following remarks should be added. (1) Although ERK1/2 and PKC-ε were not activated by isoflurane postconditioning in our study, we can not rule out some role of these kinases in mediating the protection by isoflurane, because phosphorylation was determined in whole tissue extracts, which does not consider the possible accumulation of phosphorylation at specific subcellular targets. In fact, ERK1/2 has been reported to form signaling modules with mitochondrial PKC-ε,[49] which confers cardioprotection through inhibition of the mitochondrial permeability transition pore. Also, there may be a complex time course of activation of RISKs, which we could not follow up by determining phosphorylation at a single time point. (2) We have noted increased phosphorylation of eNOS and p70S6K in a PI3K-dependent manner. Therefore, further studies are required using specific blockers to test whether these changes are only epiphenomena or active components of the protection. (3) Future studies should evaluate the optimal dosing of volatile anesthetics to obtain maximal protection during reperfusion. With this regard, it is interesting to note that Obal *et al.*[26] obtained maximal protection after only a 2-min period of sevoflurane application immediately at the onset of reperfusion. Any prolongation of sevoflurane administration rather decreased the protection. (4) As always pertinent to animal experiments, no direct extrapolation into the clinical setting should be made.

Conclusions

Using a highly controlled experimental model of postinfarct rat hearts, we were able to show that recruitment of PKB/Akt signaling at early reperfusion is a universal cardioprotective strategy, which is functional not only in healthy but also in the remodeled myocardium. This is the first demonstration to show that pharmacologic postconditioning by isoflurane retains its profound protection in severely diseased myocardium.

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